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EFFECTS OF MULTIPLE STRESSORS ON THE DYNAMICS OF A FUNGAL PATHOGEN ASSOCIATED WITH GLOBAL AMPHIBIAN DECLINES

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Submitted to the Graduate Faculty of

the Kenneth P. DietrichSchool of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

KENNETH P. DIETRICH SCHOOL OF ARTS AND SCIENCES

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September 19, 2011

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EFFECTS OF MULTIPLE STRESSORS ON THE DYNAMICS OF A FUNGAL PATHOGEN ASSOCIATED WITH GLOBAL AMPHIBIAN DECLINES

Maya Groner, Ph.D.

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Within the biological sciences there is increasing interest in understanding how ecological context alters host-pathogen interactions. Incidences of emerging infectious diseases (EIDs) are increasing around the globe with devastating impacts on many taxonomic groups. This trend is thought to result, in part, from increased frequency or intensity of interactions with stressors that facilitate the spread of a pathogen or increase its virulence. The mechanisms underlying these interactions are poorly understood.

This thesis explored how the stress of predators, competitors, and the insecticide malathion alter interactions between the fungal pathogen (*Batrachochytrium dendrobatidis*, Bd) and two of its amphibian hosts (*Rana sylvatica* and *R. pipiens*). Malathion is a widely-used insecticide that has been implicated amphibian declines. I examined how these interactions altered traits associated with within-host interactions (immune responses) and between-host interactions (life history, survival and behavior).

More than 200 amphibian species suffer from the EID caused by Bd, and the results of this study suggest that natural environmental stressors can alter patterns of infection. Sublethal exposure to predators caused decreases in releases of antimicrobial peptides (AMPs) from the skin. AMPs are an important defense against infection. The extent of this effect depended on the timing of exposure to predators as well as the length of exposure. In addition, AMPs were also more concentrated in high competition environments. This may be an adaptive response to

increased risk of infection by pathogens with density-dependent transmission. Predation would also be expected to reduce disease incidence. Tadpoles infected with Bd were more active than uninfected and resistant tadpoles, suggesting that they would be more susceptible to predation by visually-cued predators. In contrast, resistant tadpoles were the least active, making them most likely to avoid detection by predators. While the pesticide malathion had no effect on susceptibility to Bd infection or antimicrobial peptide releases, a separate study showed that it and other pesticides in the same class (acetylcholine esterase inhibitors) can alter survival and life history trajectories of amphibians through direct toxic effects and indirectly through trait- and density-mediated trophic cascades. Overall these results suggest that environmental context can have complex effects on host-pathogen interactions.

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PREFACE

Completing a dissertation would not have been possible without a substantial amount of support from within and outside of the scientific community. I owe a lot of thanks to my thesis committee. Andy Blaustein, thank you for welcoming me into your lab for three weeks when I was beginning my dissertation. I learned from you and your students many valuable skills for culturing, measuring and safely working with pathogens. The confidence that you had in me as a first year graduate student was critical at that time. Jeff Lawrence and Brian Traw, you worked as a great pair on my committee. Brian, you could see the best parts of my work, while Jeff, you helped me to find the weaker ones. I thank you both. Steve Tonsor, I can't thank you enough for all of the hours you have listened to me share my ideas and concerns about statistics, experimental design, the philosophy of science, ecological literacy, advocacy, poetry, politics, music and much more. Your encouraging words kept me from giving up more than once and you have given me a model for a level of involvement, compassion and honesty that I would like to maintain in science and in life.

Several other professors were also instrumental in this process. Much of my dissertation would not have been possible without the help of Dr. Louise Rollins-Smith. Thank you for trusting that I could measure frog skin peptides before meeting me, for quick and thoughtful answers to the numerous emails I sent you, for inviting me three times to come work and learn in your lab at Vanderbilt and for visiting us at the Pymatuning Lab of Ecology. Your generosity

with your knowledge, time and your thoughtful commentary taught me what it meant to be in a scientific community. Additional thanks to Laura Reinert for extensive training on various components of isolating, measuring and characterizing both antimicrobial peptides and DNA. Thank you John Hempel and Mark Bier for training me to characterize peptides. This is not easy to do and your patience and generosity of time and spirit were most helpful. Other professors at Pitt who were not on my committee were also helpful in my growth as a student. In particular, I have to thank Sue Kalisz, Jon Boyle, Walt Carson, Tia-Lyn Ashman and Tony Bledsoe.

The Relyea lab has a unique gestalt, full of inappropriate humor, donuts, and a lot of hard work. Rick, your advising has allowed me to finish with a dissertation I am proud of. Thank you for your guidance in grant and paper writing, experimental design, efficiency of running labs, experiments and emails, and most of all for allowing me to pursue a thesis that was full of so many unknowns. Thank you Christine for always being so warm and keeping me and the rest of the lab well fed. My labmates, Josh Auld, Jason Hoverman, Aaron Stoler, Heather Shaffery, Jessica Hua, Will Brogan, RJ Bendis, John Hammond and Rickey Cothran have been a great sounding board for ideas, last-minute editing, soccer games and a lot of silliness. Thank you. Quite a few undergraduates and one very special technician passed through the lab while I was there. In particular, I need to thank for their help and comic relief Devin Jones, Kate Henderson, Abhinav Mithal, Tim Schwartz, Caitlin Newcamp, Catherine Giancola, Dave Schmidenberg and Andy Stiff. Special thanks to Sarah Papperman for assisting in an incredible and challenging summer of field work in the Emigrant Wilderness.

The graduate students in the biology department at the University of Pittsburgh are incredibly generous with their time and thoughts. Thank you especially to Cassie Majetic, Tarek Elnaccash, Marnin Wolfe, Tom Pendergast IV, Alison Hale, Hao Ji, Sasha Rohde, Nathan Brouwer, Eric Griffin, Chris Heckel, John Paul, April Randle, Jean Deo and Henry Schumacher. Additionally, Rachel Spigler and Jill Anderson (though you were not officially at Pitt) contributed a lot of thoughtful insights to my work. I learned from all of you in the PEER discussion groups, hallway conversations and seminar presentations that we shared.

My friends and family have been supportive during this process. Thank you for understanding when field work interfered with vacations, when schedules were planned relative to wood frog breeding and for listening when I was frustrated or consumed by this process. Thank you Uncle Leonard for letting me use your house as a base for field work and for being my assistant for a few (mosquito-filled) days in the Sierra Nevadas. To my friends, thank you for providing me with the breaks that I needed, the support to finish and reminding me that science is not everything.

Throughout this process, I have been inspired by the female scientists who have served as such strong role models for me as a scientist and as a person. Balancing the challenges of a career in science, being part of a tight family and friends, and maintaining a fresh perspective is hard to do. Sonia Sultan, Kate Macneale and Beth Sanderson have been incredibly inspiring and I hope to follow in your footsteps. I dedicate this thesis to women pursuing sciences. I also dedicate this thesis to my amazing sister Anya Groner. Thanks for believing that I can do anything and for finding the humor in nearly every situation.

This work was funded by a predoctoral fellowship from the national science foundation and smaller grants from the McKinley and Pape funds, the Chicago Herpetological Society, the

American Philosophical Society, the North American Benthological Society, Sigma Xi and the American Society of Icthyology and Herpetology. Thank you for supporting graduate student research and making my thesis possible.

1.0 INTRODUCTION

Understanding how environmental variation affects outcomes of disease at the individual, population and community level is a growing interest in ecology (Hawley and Altizer 2011, Martin et al. 2011). While several technological and logistical advances have made this research more accessible outside of the laboratory, perhaps the most driving influence on the growth of these fields are trends in disease rates around the world (Daszak et al. 2000, Jones et al. 2008). Across the globe emerging infectious diseases are increasing in number, range and the intensity of their impacts (Daszak et al. 2002, Jones et al. 2008).

The impacts of EIDs as well as these complexity of their causes are poignantly illustrated in amphibians, where over 40% of all species are experiencing population declines (Stuart et al. 2004). Some of these are due to emerging infectious diseases, in particular chytridiomycosis, which is caused by infection by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd, reviewed in Daszak et al. 2003, Pounds et al. 2006, Skerratt et al. 2007, Fisher et al. 2009, Blaustein et al. 2011). Correlations between environmental variables and population-level responses to disease also suggest that environmental stress may be contributing to these patterns. Such patterns have led several authors to suggest that environmental immunosuppression (a within-host trait) may in fact be explaining the heterogeneity in infection prevalence and population-level responses to infection (a between-host phenomenon) (Carey et al. 1999, Hayes et al. 2010, Voyles 2010, Blaustein et al. 2011, Rollins-Smith 2011). The goal of this thesis is to

understand interacting effects of several environmental stressors on infection rates and immune function and also to understand how infection alters how organisms interact with these stressors. In addition to Bd have chosen to focus on several stressors in this study (predators, competition and acetylcholine esterase-inhibiting pesticides) because they are all common, have heterogeneous distributions are known to interact with each other and have well-characterized physiological, behavioral and life historical effects on anurans (e.g. Relyea 2003c, Relyea 2004a, b, Relyea and Diecks 2008).

The first two experiments focus on characterizing the direct and indirect effects of common insecticides on amphibians in the presence and absence of Bd. Acetylcholine esterase (AchE) inhibitors are widely used insecticides and their usage has been correlated with amphibian declines (Davidson et al. 2001, 2002 and 2004), however amphibians are commonly exposed to concentrations far below what is considered lethal. It is still unclear how these low concentrations are contributing to these declines. I have addressed this topic with two experiments.

Chapter two focuses on the community-level effects of AchE inhibiting insecticides. Past research in the Relyea lab demonstrates that these chemicals can have dramatic indirect effects on amphibian populations as a result of trophic cascades (e.g. Relyea and Diecks 2008). In this study I addressed the generality of this observation and examined if and when two insecticides with the same mode of action would have comparable effects on pond communities. We found that both insecticides affected pond communities through two trophic cascades, a density-mediated cascade stemming from a decrease in zooplankton as a result of direct toxicity and a behavioral cascade resulting from decreased activity that was coupled with, in some cases, decreased tadpole populations. Despite acting through similar mechanisms, variation in the

timing and strength of these cascades at different concentrations and with different pesticides resulted in different community-level effects. This paper is published in Freshwater Biology (Groner and Relyea 2011). Rick Relyea is a co-author.

A second mechanism through which 'sublethal' levels of AchE inhibiting insecticides may be influencing amphibians is by immunomodulation. These chemicals have been shown to be immunosuppressive to the acquired immune system (reviewed in Galloway and Handy 2003), but little is known about their effects on the innate immune system, which is thought to be important for resistance against Bd. Previous studies in the Relyea lab have shown that pesticides can be more toxic to tadpoles when they combined with a second stressor, such as predation or competition (e.g. Relyea 2003c, 2004b, 2005, 2006, Relyea and Hoverman 2008). Because I thought this same synergism might exist for immunosuppressive effects of pesticide exposure, I examined the effects of pesticide exposure in the presence and absence of caged tadpole predators. Specifically I examined whether malathion, a common insecticide in North America, affected the production of antimicrobial peptides (AMPs) in wood frog skin in the presence and absence of predator cues. These peptides are thought to be an important innate immune defense against skin infection and some of them have been shown to inhibit *in vitro* growth of Bd (reviewed in Rollins-Smith and Conlon 2005, Rollins-Smith 2011). I also tested if these treatments altered susceptibility to Bd in order to determine if immunosuppressive effects of these stressors was correlated with increased disease susceptibility.

While, several hypotheses have suggested a link between pesticide exposure, immune function and disease susceptibility in frogs, we failed to find support for this hypothesis. If malathion does suppress AMPs or increase disease susceptibility these effects must operate on shorter time scales or within different ontogenetic windows. We did find the intriguing result that

exposure to predators decreased production of AMPs, but increased post-metamorphic survival, suggesting that this stressor might decrease fitness in the short term, but increase it in the long term. This work was a collaborative effort. Rick Relyea helped with the experimental design, Louise Rollins-Smith and Laura Reinert helped with AMP extraction and quantification, John Hempel and Mark Bier trained and assisted me with mass spectrometry and characterization of peptides. Andy Blaustein, Julia Buck and Stephanie Gervasi performed Bd challenge experiments. All co-authors helped edit drafts of this paper. This paper will be submitted to Ecological Applications.

The length of an exposure to a stressor and the ontogenetic stage of the organism that is exposed to the stressor can have large effects on the physiological response to stress (Martin 2009). This variation is ecologically relevant as many environmental stressors have heterogeneous distributions across space and time. Chapter three demonstrated that predator-cues could alter the production of antimicrobial peptides in anurans. In this chapter, we explored the conditionality and generality of this effect. We exposed tadpoles of another Ranid species, wood frogs (*Rana sylvatica*) to caged predators early in development, late in development, chronically or not at all. Since past studies in the Relyea lab have shown that inducible defenses against predators are often lower at high competition (e.g. Relyea 2004a, Relyea and Auld 2005), we crossed these treatments with a low and a high competition treatment. Our results show that the timing and duration of these stressors matter.. AMP production increased in high competition, but only in some treatments. Early exposure to predators and chronic exposure to predators caused smaller or no increases in AMPs in high competition environments. This paper will be submitted to Functional ecology. Rick Relyea, Louise Rollins-Smith, Laura Reinert and John Hempel are co-authors.

While the past studies showed that environmental stressors can alter traits that mediate host-parasite interactions, the reciprocal interactions may also occur; resisting or tolerating an infection may influence how an organism responds to other environmental challenges. This can occur because many immune responses are metabolically costly and immune activity can cause trade-offs on other physiological or behavioral traits that may defend an organism against other threats (e.g. Sheldon and Verlhurst 1996, reviewed in Hawley and Altizer 2011). In chapter five we addressed this topic by testing how exposure to Bd alters inducible defenses of wood frog tadpoles against predators and how exposure to predators alters the prevalence and intensity of Bd infections in tadpoles. Because we had both resistant and tolerant tadpoles, we also evaluated whether these two strategies incurred different costs on the expression of inducible predator defenses. We found that exposure to Bd increased susceptibility to predation for infected tadpoles (by altering behavioral inducible defenses), but decreased susceptibility for resistant tadpoles. Exposure to predators decreased infection intensity, but did not alter the proportion of tadpoles that because infected. Collectively these results suggest that predators should reduce overall infection prevalence and intensity in tadpoles. This paper will be submitted to Ecology Letters. Rick Relyea is a co-author.

Chapter 6 discusses some of the implications of this thesis for the expanding fields of disease ecology and eco-immunology as well as future research directions.

2.0 A TALE OF TWO PESTICIDES: HOW COMMON INSECTICIDES AFFECT AQUATIC COMMUNITIES

2.1 ABSTRACT

Recent ecotoxicology studies show that pesticide exposure can alter community composition, structure, and function. Generally community responses to pesticides are driven by trait- and density- mediated indirect effects resulting from sublethal and lethal effects of pesticide exposure on vulnerable taxa. These effects depend upon the concentration of the pesticide and the frequency of exposure. While more research is needed to understand community-level responses pesticide exposure, testing the effects of multitudes of registered chemicals on ecologically relevant communities is overwhelming. Recent reviews suggest that contaminants with similar modes of action should produce comparable community-level responses because they have similar direct effects and, as a result, similar indirect effects. This hypothesis remains largely untested.

We subjected pond communities (containing zooplankton, phytoplankton, periphyton, and leopard frog tadpoles (*Rana pipiens*)) to several applications (single applications of medium or high concentrations or weekly applications of a lower concentration) of two acetylcholine esterase inhibiting insecticides, malathion and carbaryl that have comparable toxicity for aquatic organisms. We found that both insecticides cause comparable trophic cascades that affect

zooplankton and phytoplankton abundances; however their effects on amphibians diverged, especially when exposed to higher concentrations of insecticides. Malathion caused a trophic cascade beginning with a decline in cladocerans followed by increases in phytoplankton. At a medium concentration, this cascade also caused a subsequent decrease in periphyton. Carbaryl caused a similar trophic cascade with the highest application, a weak trophic cascade with the medium application and no cascade with smallest application. Malathion directly reduced tadpole survival at all concentrations. Survivors in the two higher treatments were larger at metamorphosis while survivors in the lowest treatments were smaller and developed slowly. In contrast, carbaryl was not directly toxic to tadpoles, but indirectly reduced survival because slow growth and development prevented some tadpoles from metamorphosing before the mesocosms dried at medium and low applications. These results suggest that these common pesticides, which share the same mode of action, have similar effects on zooplankton and algae, but differences in the strength and timing of their effects on tadpoles reduce the generality of responses at higher trophic levels. Overall, general predictive models of contaminant effects could be improved by incorporating the relative timing of direct and indirect effects of exposure.

2.2 INTRODUCTION

Understanding how anthropogenic contaminants affect ecological communities is a major modern challenge. We face the task of understanding the effects of nearly 80,000 registered contaminants in the U.S., including hundreds of pesticide active ingredients that come in thousands of commercial formulations (Jones et al. 2004, Giagnessi and Reiger 2006a, b). Moreover, the effects of contaminants on communities depend upon many factors including the

concentration of the contaminant, the timing of the exposure, and the number of exposures (Relyea and Diecks 2008).

Recent reviews have suggested that many impacts of contaminants on communities can be predicted by applying the same conceptual framework used to understand community composition and structure in uncontaminated systems. In particular, knowledge of direct lethal and sublethal effects of contaminants and the density- and trait-mediated indirect effects that they cause provides a useful framework for making predictions about changes in community structure (Van den Brink et al. 2002, Fleeger et al. 2003, Traas et al. 2004, Relyea and Hoverman 2006, Rohr et al. 2006, Clements and Rohr 2009). This community ecology approach to ecotoxicology suggests that pesticides with similar toxicity and modes of action should have comparable effects on communities even when contaminants are in different chemical classes.

While many reviews have suggested these similarities, there are few direct tests that examine the effects of similar contaminants on communities (but see Relyea 2005a, Boone and Bridges-Britton 2006, Relyea 2009) and fewer that make comparisons across several application regimes (Farmer et al. 1995, Williams and Semlitsch 2009). Different application regimes of pesticides yield diverse results. For example, Relyea and Diecks (2008) found that a press treatment consisting of seven weekly applications of 10 μg/L of malathion caused larger impacts on many of the response variables than single pulse applications that were 25 times higher in concentration. It would be useful to know if such patterns are consistent among similar pesticides; however, finding such patterns by comparing studies across the literature is challenging as studies vary widely in community assemblage, concentrations of contaminants used and the timing and frequency of applications. Thus, experiments comparing the roles of contaminants at several concentrations and application regimes are needed to test if and when

generalizations can be made about their community effects.

Among contaminants, pesticides have received recent attention for their potential to alter population and community dynamics (Relyea and Hoverman 2006). For instance, pesticides have been implicated as a contributor to amphibian declines (Blaustein et al. 2003). The strongest data suggesting a role of contaminants in declines are from California in which a correlation exists between declining populations that are downwind of applications of carbamate and organophosphate insecticides that inhibit acetylcholine esterase (AchE; Davidson et al. 2001, 2002). Moreover, Pacific tree frogs (*Psuedacris regilla*) with decreased AchE activity are found in sites where co-occurring species are declining (Sparling et al. 2001); however, the pesticides in these wetlands are found at sublethal concentrations based on traditional short-term laboratory experiments that are designed to determine the lethal concentrations that kills 50% of a population (i.e. LC50 tests). This suggests that these pesticides may act in ways other than through direct toxicity (e.g. Relyea and Diecks 2008).

The goal of this study was to test whether pesticides with similar modes of action have similar impacts on aquatic communities and which application regimes produce these similarities. We chose to use the insecticides malathion and carbaryl because they share the same mode of action (i.e. AchE inhibitors), are in different chemical classes, are among the most commonly used insecticides in the United States (Kiely et al. 2004) and have been associated with amphibian declines (Davidson et al. 2001, 2002). We tested the affects of each pesticide using a low concentration applied weekly, a medium concentration applied once and a high concentration applied once. Based on LC50 values for various taxa and knowledge of trophic interactions within this community, we hypothesized that the two insecticides would cause similar direct and indirect effects on aquatic communities. Specifically, we predicted that 1)

malathion and carbaryl would both kill zooplankton, 2) reductions in zooplankton would cause a trophic cascade in which phytoplankton would increase and subsequently shade periphyton, 3) as a result of decreased periphyton (the tadpole's main food source), tadpole growth and development would be reduced, and 4) low, weekly applications of the insecticides would cause a stronger trophic cascade than larger, single applications, although the cascade could take longer to develop. While other studies have examined community effects across a range of pesticide applications, none have made comparisons across such a large range with more than one pesticide (Relyea and Diecks 2008).

Pesticide background

Both carbaryl and malathion are widely used broad-spectrum insecticides that act by inhibiting AchE. Both insectides act by binding to AchE, thereby disrupting nervous impulses and chemical signaling at neurotransmitters. Concentrations of each chemical that are toxic to amphibians are quite similar, so comparisons can be made without adjusting for toxicity.

In the United States, a total of 1.8 million kg of carbaryl, a carbamate insecticide, is sold annually. Approximately 50% goes to agricultural uses (e.g. tomatoes, oranges, alfalfa, rice) and another 50% to non-agricultural uses (e.g. gardening, pet care), making it the second most common insecticide sold for non-agricultural use (Kiely et al. 2004). Application intervals are between 3 and 14 d depending on use (Garber et al. 2007). Carbaryl reaches water bodies through run-off and aerial deposition. Peak expected environmental concentrations (EECs) for carbaryl in aquatic systems in maximum use scenarios range from 0.47 to 166 ppb, although spraying for rice has an EEC of 2579 ppb (Garber et al. 2007). United States national water quality assessment program surveys have found carbaryl in nearly 50% of water bodies surveyed

with maximum concentrations of 1.06 ppb (Garber et al. 2007). The breakdown rate of carbaryl in water is pH-dependent; it degrades in distilled water with a half-life of 3 hrs at a pH of 9 and 12 d at a pH of 7 (Wolfe et al. 1978). Estimates of lethal concentrations for larval anurans (LC50s) vary between 2.5 and 20.6 ppm (Marian et al. 1983, Relyea 2003c).

Malathion is an organophosphate insecticide, which, unlike carbaryl, binds irreversibly to AchE. In the United States, it is the most commonly used insecticide, with 9 to 11.3 million kg of active ingredient used annually in the agricultural sector (Kiely et al. 2004). Malathion is also used for gardening and public health pest-control programs. California records show that it is sprayed every month of the year with 2- to 14-d intervals between applications (Odenkirchen and Wente 2007). It reaches water bodies indirectly via run-off and aerial transport and directly via application to water bodies to control potential mosquito vectors of disease and pests of aquatic crops (Odenkirchen and Wente 2007). The U.S. Environmental Protection Agency's EECs for malathion in surface water range from 9 to 27 ppb when sprayed on terrestrial crops and 1404 to 1797 ppb when sprayed on aquatic crops (rice and watercress, Odenkirchen and Wente 2007). Breakdown rates of malathion increase with temperature and alkalinity; it has a half-life greater than 4 yrs at a pH of 4. At a pH of 8, malathion has a half-life of 1 hr at 40 °C and a half-life of 40 d at 0 °C (Wolfe et al. 1977). Malathion is highly toxic to aquatic invertebrates and moderately toxic to amphibians with LC50 values for larval anurans between 1.3 and 5.9 ppm (Relyea 2004b, USEPA Ecotox database, *available online* http://cfpub.epa.gov/ecotox/). Malathion transforms via oxidative desulfuration into malaoxon, which is 100 times more lethal to foothill yellow-legged frogs (*Rana boylii*) than malathion itself (Sparling and Fellers 2007).

2.3 METHODS

2.3.1 Experimental Design

The experiment was conducted at the University of Pittsburgh's Pymatuning Lab of Ecology (Linesville, Pennsylvania, USA). We used a completely randomized design employing seven treatments. In addition to a 0 ppb control, each pesticide was applied at nominal concentrations of 25 ppb applied weekly, 250 ppb applied once or 2,500 ppb applied once. Hereafter, we refer to these treatments as "control," "weekly low," "single medium," and "single high". Since we were interested in both direct and indirect effects of pesticides on amphibians, we chose treatment concentrations that ranged from sublethal to lethal for many amphibians (Relyea 2004b).

Each of the seven treatments was replicated four times for a total of 28 experimental units. The experimental units were 1200-L mesocosms filled with approximately 1,055 L of well water between 21 and 23 April 2007 and covered with 60% shade-cloth lids to prevent colonization by ovipositing animals and escape by metamorphosing frogs. Oak (Quercus spp.) leaf litter (300g) and rabbit chow (25 g) were added to each tank on 30 April to provide nutrients and substrate to the mesocosms. Aliquots of water collected from several local ponds were

screened for predators and added on 1, 4, and 24 May to provide a natural source of algae and zooplankton to the mesocosms. Two unglazed clay tiles (225 cm2) were added to all tanks on 7 and 10 May to serve as periphyton samplers. All tiles were positioned vertically on the north side of each mesocosm.

Leopard frogs were collected from one egg mass located in northwest PA and hatched in covered 200-L wading pools. While we typically would use tadpoles from more than one egg mass to increase genetic diversity, we were unsuccessful in locating additional egg masses in 2007. On 24 May, 40 tadpoles (initial mass 59.3 ± 2.4 mg) were added to each tank. This density of leopard frog tadpoles was used to ensure competition for resources (periphyton; Relyea and Diecks, 2008). We examined handling stress on the tadpoles by holding 20 individuals for 24 hrs. The resulting survival was 100%. Tadpoles in mesocosms were allowed to recover from handling for a week before applying the pesticide treatments.

Pesticides were first applied to the mesocosms on 30 May (defined as day 1 of the experiment). For the weekly low applications, we reapplied the pesticides after weeks 1, 2, 3, and 4. Weekly low applications were stopped after week 4 because frogs began to metamorphose. To achieve the nominal pesticide concentrations, we added 52.75 uL, 527.5 uL and 5,275 uL of commercial grade malathion (50% active ingredient, Malathion Plus; Ortho Corp. Marysville, Ohio, USA) and 117.2 uL, 1,172 uL and 11,722 uL of commercial grade carbaryl (22.5% active ingredient, GardenTech Sevinâ Ready -to-Spray Bug Killer; Aventis Cropscience, Research Triangle Park, North Carolina, USA) to the appropriate mesocosms. To ensure that pesticides were mixed into the treatments, we stirred each mesocosm for several minutes after adding pesticides and then we allowed several hours to pass before collecting samples for testing. Past experiments have confirmed that carbaryl and malathion degrade quickly in mesocosms such that

weekly applications at low concentrations do not increase the total concentration of pesticides over time (Boone and Semlitsch 2002, Relyea and Diecks 2008). Water samples were collected from all mesocosms 4 hrs after pesticide treatments were applied and pooled across replicates. Samples were stored at 4 °C and later shipped for analysis by an independent lab using high-pressure liquid chromatography (Mississippi State Chemical Laboratory, MS).

Of the six water samples sent for testing, two samples were damaged during shipment (250 ppb malathion and 2,500 ppb carbaryl). The two tested malathion samples had nominal concentrations of 25 ppb and 2500 ppb but actual concentrations of 3.1 ppb and 384 ppb, respectively. The two tested carbaryl samples had nominal concentrations of 25 ppb and 250 ppb but actual concentration of 13.5 ppb and 141 ppb, respectively. Thus, compared to the nominal concentrations, the actual concentrations were 54-56% for carbaryl and 12-15% for malathion. Based on these measured concentrations, we estimate the actual concentration of the two damaged samples to be approximately 34 ppb (for the 250 ppb nominal malathion treatment) and 1380 ppb (for the 2500 ppb nominal carbaryl treatment). While actual concentrations deviated considerably from the nominal concentrations, the deviation was consistent among tested concentrations within each pesticide. Variation from nominal concentrations is frequently found in mesocosm experiments (reviewed in Brock et al. 2000) and is thought to arise from pesticide precipitation, volatilization, binding to substrates, or degradation of stored samples before mailing (Farmer et al. 1995, Brock et al. 2000); thus low measured values do not necessarily reflect error in the application of the pesticide.

2.3.2 Response Variables

To quantify the responses of primary producers and consumers to the treatments, we sampled periphyton, phytoplankton, and zooplankton on days 7 and 21. Periphyton was collected by scrubbing one side of an unglazed ceramic tile into a tub of filtered well water. We then vacuum-filtered the slurry onto a pre-dried (80°C) and pre-weighed Whatman GF/C filter. The filter was dried again at 80°C for 24 hrs and weighed to determine the biomass of periphyton that had grown on the tile.

Phytoplankton density measured as chlorophyll *a* (chl *a*) was quantified by collecting 250 mL of water at the bottom and top of the water column in the center of each mesocosm. Water was vacuum filtered onto a Whatman GF/C filter. Filters were wrapped and frozen at -80°C. Chl *a* was extracted with dimethyl formamide, and then quantified using a fluorometer (Model TD-700, Turner Designs, Sunnyvale, CA, USA). Chl *a* concentrations were adjusted for breakdown products (pheophytin) which were also quantified with a fluorometer.

Zooplankton abundance was quantified by collecting 200 ml of water from five standardized locations within each mesocosm using a tube sampler plunged into the water column. The sample was filtered through a 62 μm Nitex screen and the zooplankton were preserved in 70% ethanol. Zooplankton were subsequently identified and counted using a dissecting scope. Past studies using the same zooplankton assemblages have found that sensitivity to insecticides differs between cladocerans and copepods, but sensitivity is very similar among species belonging to each group. As a result, we only identified zooplankton to the level of cladocerans and copepods (Relyea and Diecks 2008).

Once the first tadpole metamorphosed on day 31, we added pieces of wooden lath to each mesocosm to serve as perches for metamorphs. From this date onward, we checked for

metamorphs daily and collected all individuals that had both forelimbs emerged (Gosner stage 42; Gosner 1960). We considered metamorphosis complete when the tail was resorbed to less than 3 mm. All collected individuals that were not fully metamorphosed were placed in 1-L plastic containers with wet sphagnum moss and checked daily until metamorphosis was complete. Each individual was weighed after completing metamorphosis. For each tank, the mean time to metamorphosis, mean mass at metamorphosis and survival to metamorphosis were used as our amphibian response variables.

Pond drying is a common phenomenon in habitats where leopard frogs breed. Tadpoles detect pond drying via reductions in water volume and consequently increase their development rate in order to metamorphose before the pond dries (Denver 1998). To examine how tadpoles exposed to pesticides respond to pond drying, we removed 225 L of water from each mesocosm every 3 d beginning on day 88, 60 days after the last weekly pesticide application. Drying was completed on day 97. Tadpoles that had not metamorphosed by day 97 were preserved in ethanol and considered as mortalities since they would not have survived in a dry pond. Individuals with at least one emerged forelimb were considered survivors and held until metamorphosis was complete (e.g. Relyea and Diecks 2008). The remaining tadpoles were counted, so that we could differentiate between individuals that died due to slow development and pond drying from tadpoles that died during the experiment.

To determine abiotic conditions and effects of treatments on water quality, we measured dissolved oxygen (DO), temperature, pH and light extinction. Dissolved oxygen, pH and temperature were measured between 1100 and 1300 hours on days 7 and 20 with a calibrated Wissenschaftlich-Technische Werkstatten MultiLine P4 meter. Light extinction was measured on days 7 and 21 to quantify the shading effect of phytoplankton. We measured photosynthetic

active radiation at 10 cm and 30 cm below the surface using a photometer (LI-COR, Lincoln, Nebraska, USA). We calculated light extinction (k) using the formula:

$$K = \frac{\ln (L_{10}/L_{30})}{d}$$

where L_{10} and L_{30} are the photons detected at 10 cm and 30 cm respectively and d is the difference in depth between the two measurements.

2.3.3 Statistical Analyses

The data were analyzed with one-way analyses of variance (ANOVA). Given that water quality data (pH, dissolved oxygen, temperature and light extinction) and biotic data (periphyton mass, chl *a*, cladocerans and copepod) were measured at two time points, we initially analyzed each response variable with separate repeated-measure ANOVAs (rm-ANOVAs). Since most response variables had significant time-by-treatment interactions, we then conducted multivariate one-way ANOVAs (MANOVAs) on the abiotic variables within each time point.

A separate one-way MANOVA was used to analyze amphibian data (leopard frog mass at metamorphosis, time to metamorphosis and survival) since these data were not repeatedly sampled. One replicate (single medium carbaryl treatment) was excluded from the analysis as it was colonized by libellulid dragonfly larvae late in the experiment and had much higher mortality of tadpoles than other replicates of this treatment. This treatment was not excluded from the other analyses, because those data were collected prior to colonization by the dragonflies.

Where necessary, response variables were log- or arcsine-transformed to meet the assumption of normality. For the water quality, algae and zooplankton data, the assumption of

homogeneity of variances was met for all data except the cladoceran and periphyton data for the first sample date and copepods and light extinction data for the second sample date. For the amphibian data, all response variables except for mass at metamorphosis met the assumption of homogeneous variances. ANOVAs are robust to violations of only one assumption (Quinn and Keough 2002, p. 191). When evaluating MANOVAs, we used Pillai's trace, which is robust to violations of the assumption of homogeneous variances (Quinn and Keough, 2002, p. 434).

Whenever there were significant main effects in MANOVAs, we performed ANOVAs on each response variable. When there were significant ANOVAs, pairwise comparisons were made between pesticide treatments and the control using Fisher's LSD test. All pair-wise comparisons were two-tailed, with the exception of amphibian survival.

2.4 RESULTS

2.4.1 Water Quality

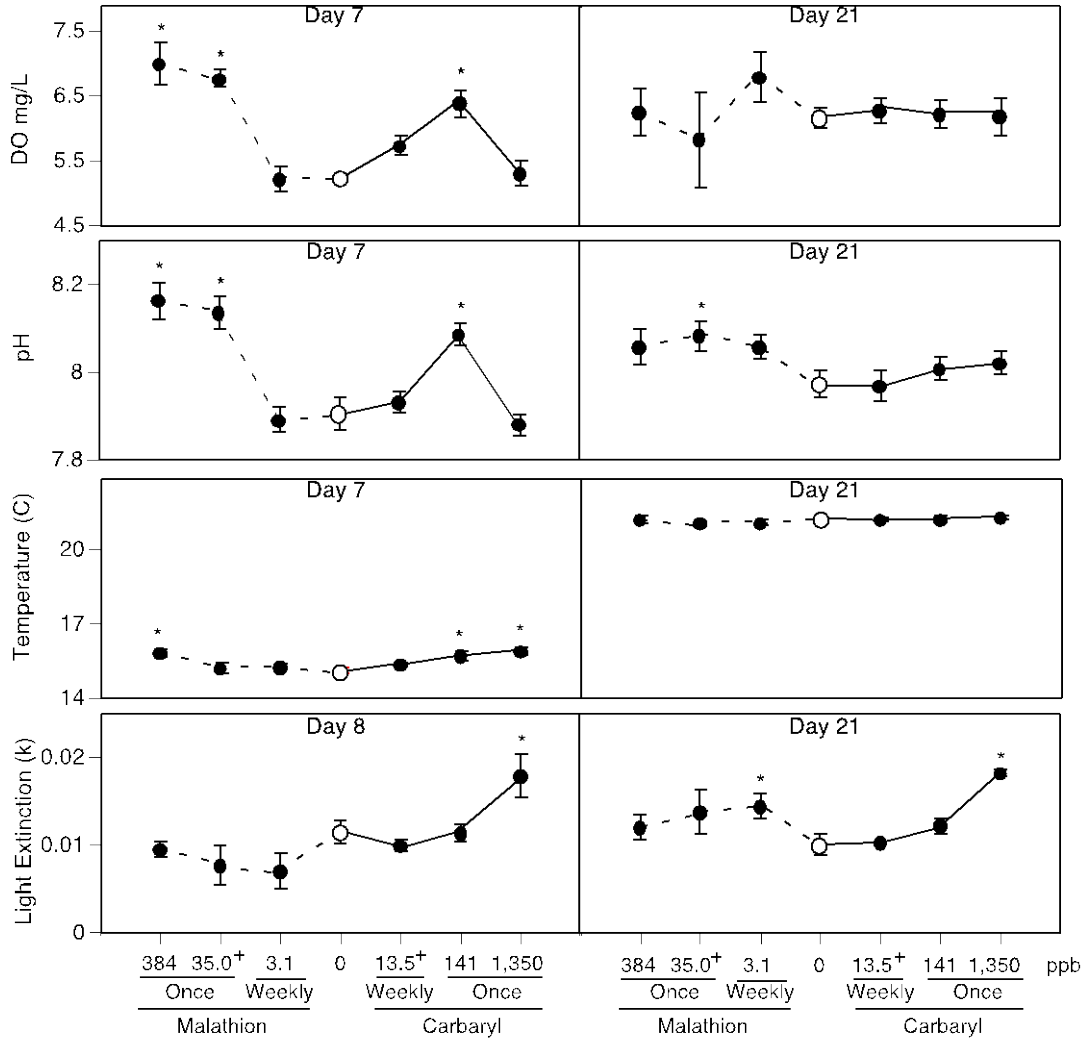


Figure 2.1. The effects of pesticide concentration, type (carbaryl or malathion), and timing of application (delivered weekly or once) on water dissolved oxygen, pH, temperature and light extinction 7 or 8 days and 21 days after the experiment was initiated. Data are means ± SE. Asterisks indicate treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). [+]Actual concentrations were not available, values were estimated.

Water quality was affected by the insecticides on day 7 and 21. Repeated-measures ANOVAs for pH, dissolved oxygen, temperature and light extinction revealed significant (or marginally non-significant) time-by-treatment interactions on all response variables (Figure 2.1, Table 2.1). As a result, we ran separate MANOVAs for each sample date and found significant multivariate effects on day 7 (Pillai's Trace $F_{18, 84} = 3.494$, $P < 0.001$) and 21 (Pillai's Trace $F_{18, 84} = 1.78$, $P = 0.029$).

The multivariate effect on day 7 was driven by all four water quality variables (Table 1, Figure 1). For malathion, the weekly low treatment did not differ from the control for any water quality variables. The single medium treatment had higher pH and DO and the single high treatment had higher pH, DO and temperature (all $P < 0.005$). For carbaryl, the weekly low treatment was not different from the control for any of the water quality variables. The single medium treatment had higher pH, DO and temperature (all $P \leq 0.009$). The single high treatment had a higher temperature and 56% greater light extinction (both $P \leq 0.012$).

The multivariate effect on day 21 was driven by light extinction and pH. For malathion, relative to the control, the weekly low treatment had 50% greater light extinction ($P = 0.032$) and the medium treatment had 40% greater light extinction, although the latter difference was marginally non-significant ($P = 0.067$). The single medium malathion treatment also had a higher pH than the control ($P = 0.025$). The single high malathion treatment did not differ from the control in any of the water quality variables. For carbaryl, only the single high treatment differed from the control in water quality variables. Mesocosms exposed to single high concentrations of carbaryl had 80% greater light extinction ($P < 0.001$).

Table 2.1. Analyses of pesticide effects on water quality response variables (pH, dissolved oxygen, temperature, and light extinction). Results are from rm-ANOVAs followed by ANOVAs conducted within each sample date. For each response variable, *F*-values are listed first followed by *P*-values in parentheses. Bold *P*-values are significant (α = 0.05).

Response variable	Treatment (df = 6, 21)	Time (df = 1, 21)	Treatment x Time (df = 6, 21)	Treatment on day 7 (df = 6, 21)	Treatment on day 21 (6, 21)
pH	13.2 (< **0.001**)	2.0 (0.177)	5.0 (**0.003**)	15.5 (< **0.001**)	1.9 (0.128)
Dissolved oxygen	2.6 (**0.049**)	3.1 (0.092)	4.5 (**0.005**)	15.2 (< **0.001**)	0.6 (0.766)
Temperature	4.9 (**0.003**)	6286 (< **0.001**)	2.4 (0.067)	4.5 (**0.005**)	0.8 (0.563)
Light extinction	4.7 (**0.003**)	13.6 (**0.001**)	4.0 (**0.008**)	4.7 (**0.004**)	4.3 (**0.006**)

2.4.2 Algae and Zooplankton

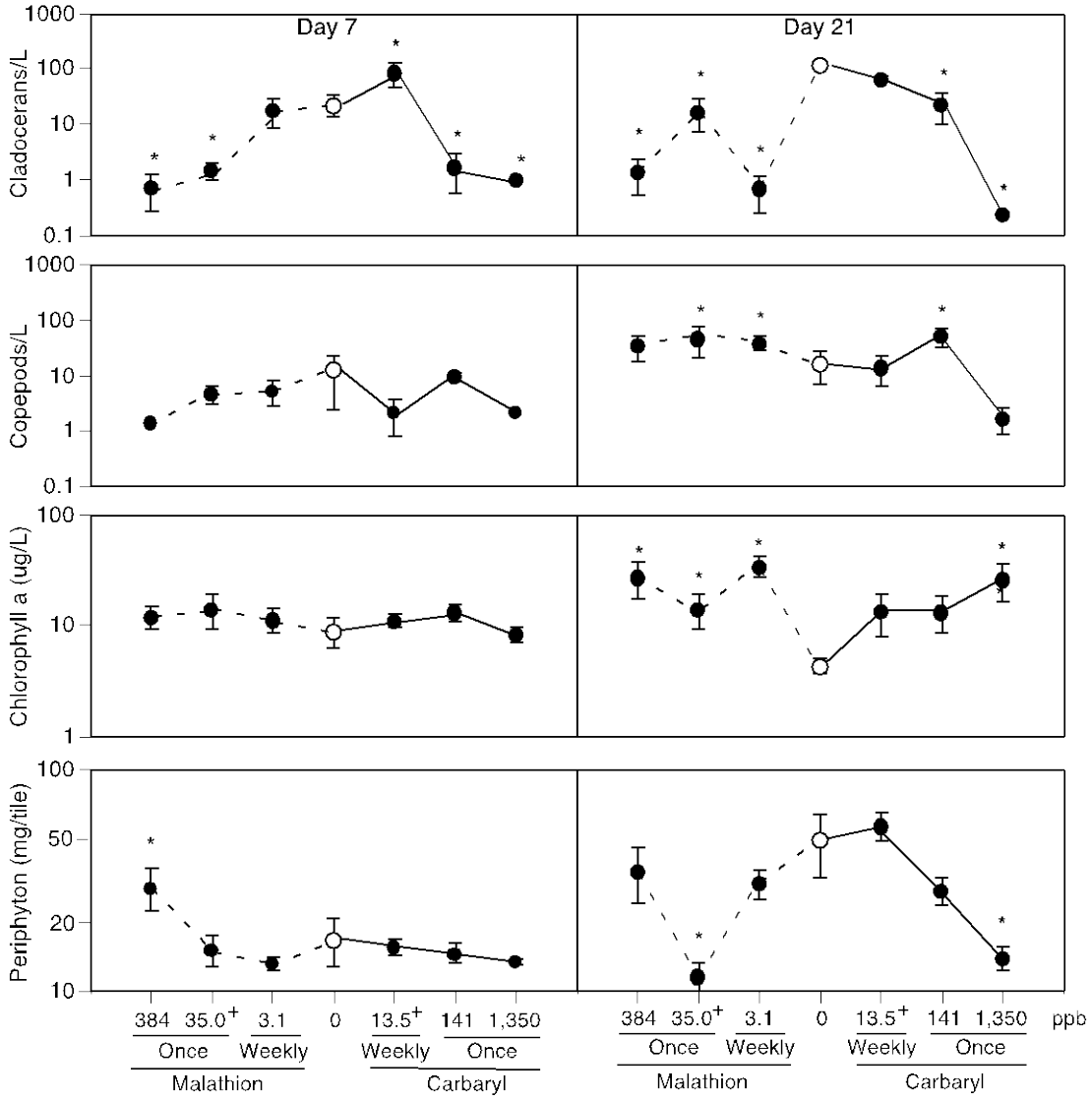


Figure 2.2. The effects of pesticide concentration, type (carbaryl or malathion), and timing of application (delivered weekly or once) on chlorophyll *a* concentration, periphyton mass and copepod and cladoceran abundances 7 days and 21 days after the experiment was initiated. Data are means ± SE. Asterisks indicate treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). $^{+}$Actual concentrations were not available, values were estimated.

Pesticide treatments affected both algal and zooplankton populations. Repeated-measures ANOVAs on cladocerans, copepods, phytoplankton (measured as chl *a*) and periphyton showed significant time-by-treatment interactions for three of the four response variables (Figure 2.2, Table 2.2). As a result, we ran a separate MANOVA for each sample date and found significant multivariate effects of treatment on day 7 (Pillai's Trace $F_{18, 84} = 2.464$, $P = 0.001$) and day 21 (Pillai's Trace $F_{18, 84} = 4.149$, $P < 0.001$).

The multivariate effect on day 7 was driven by changes in cladocerans and periphyton relative to the control (Table 2.2, Figure 2.2). For cladocerans in the malathion treatments, the weekly low treatment did not differ from the control whereas the single medium and single high treatments reduced cladocerans by 93% and 97%, respectively (both $P < 0.021$). For cladocerans in the carbaryl treatments, the weekly low treatment increased cladocerans by 380% ($P < 0.032$), while the single medium and single high treatments reduced cladocerans by 92% and 98%, respectively (both $P < 0.05$). At this early stage of the experiment, there were no effects on copepods. Periphyton biomass was only affected in one treatment; the single high malathion treatment had 20% more periphyton than the control ($P = 0.013$).

All four taxonomic groups contributed to multivariate effect on day 21 (Figure 2.2, Table 2). For cladocerans, the three malathion treatments had 86% to 99% fewer individuals than the control (all $P < 0.001$). The weekly low carbaryl treatment did not differ from the control, but the single medium and single high carbaryl treatments caused cladoceran populations to decline by 81% and 99.9%, respectively (both $P < 0.001$).

At this later point in the experiment, the pesticide treatments also affected the abundance of copepods. For malathion, the weekly low and the single medium treatments had 235% to

288% more copepods relative to the control (both $P < 0.05$). The single high malathion treatment had 216% more copepods, but this was not-significant ($P = 0.079$). For carbaryl, the weekly low treatment did not differ from the control, the single medium carbaryl treatment had 315% more copepods ($P < 0.043$), and the single high carbaryl treatment did not differ from the control ($P = 0.083$).

On day 21, all malathion treatments and the single high carbaryl treatment had more phytoplankton than the control (Figure 2.2, Table 2.2). For the malathion treatments, these increases ranged between 320% and 790% (all $P < 0.05$). For carbaryl, the weekly low and single medium treatments did not differ from the control (both $P \leq 0.111$) whereas the single high carbaryl treatment caused a 600% increase in chl *a* ($P = 0.007$).

Some of the pesticide treatments also affected periphyton biomass on day 21. For malathion, the single medium treatment reduced periphyton by 70% ($P < 0.001$). For carbaryl, the single high treatment reduced periphyton by 76% ($P < 0.001$).

Table 2.2. Results of analyses of pesticide effects on cladocerans, copepods, phytoplankton (assayed as chlorophyll a), and periphyton. Results are repeated-measures ANOVAs followed ANOVAs conducted on each sample date. *F*-values are listed first, followed by *P*-values in parenthesis. Bold *P*-values are significant at ($\alpha = 0.05$).

Response variable	Treatment (df = 6, 21)	Time (df =1, 21)	Time x treatment (df = 6, 21)	Treatment on day 7 (df = 6, 21)	Treatment on day 21 (df = 6, 21)
Cladocerans	24.2 (< **0.001**)	4.2 (0.054)	5.6 (**0.001**)	8.3 (< **0.001**)	22.3 (< **0.001**)
Copepods	5.1 (**0.002**)	28.5 (< **0.001**)	1.5 (0.225)	1.6 (0.180)	5.1 (**0.002**)
Chlorophyll *a*	1.9 (0.120)	4.5 (**0.045**)	2.6 (**0.048**)	0.5 (0.812)	3.0 (**0.029**)
Periphyton	4.0 (**0.008**)	80.0 (< **0.001**)	14.5 (< **0.001**)	4.5 (**0.030**)	7.0 (< **0.001**)

2.4.3 Leopard frogs

The effects of malathion and carbaryl on tadpole survival and life history depended upon the application amount and frequency. The MANOVA on the leopard frog response variables showed a multivariate effect of treatment (Pillai's Trace $F_{18, 60}$ = 6.028, P < 0 .001). This multivariate effect was driven by univariate effects on survival ($F_{6,27}$ = 3.390, P < 0.019), time to metamorphosis ($F_{6,27}$ = 12.156, P < 0.001), and size at metamorphosis ($F_{6,27}$ = 10.456, P < 0.001).

Exposure to pesticides affected frog survival (Figure 2.3). For malathion, relative to the control, survival was 8% lower in the weekly low and single medium treatments (both P < 0.05) and 22% lower in the single high treatment (P < 0.001). For all three treatments, nearly all the mortality occurred during the experiment; few tadpoles remained when the mesocosms were dried. For carbaryl, relative to the control, survival was 10% lower in the weekly low treatment and 22% lower in the single medium treatment (both P < 0.05) but there was no effect of the high treatment (P = 0.994). In the weekly low and single medium treatments, most of the mortality was due to animals not completing metamorphosis before the mesocosms dried. These mortalities were observed as live tadpoles that had not metamorphosed by day 97 when the drying was finished.

Time to metamorphosis was also affected by pesticide treatments (Figure 2.3). For malathion, tadpoles in the weekly low treatment took an average of 17 d longer to metamorphose (P < 0.001), while tadpoles in the single medium and single high treatments did not differ from the control. For carbaryl, relative to the control, tadpoles in the weekly low and single medium treatments took longer to metamorphose by 10 and 20 d respectively (both P < 0.01). Tadpoles in the single high treatment did not differ from the control.

Mass at metamorphosis varied with treatment. For malathion, relative to the control, tadpoles in the weekly low treatment, metamorphosed with 15% lower mass ($P = 0.011$). Conversely, tadpoles in the single medium and single high malathion treatments metamorphosed with 13 to 15% greater mass (both $P < 0.018$). For carbaryl, tadpoles in the weekly low and single medium treatments metamorphosed with 12% less mass (both $P < 0.05$), while tadpoles in the single high treatment had similar mass to the control.

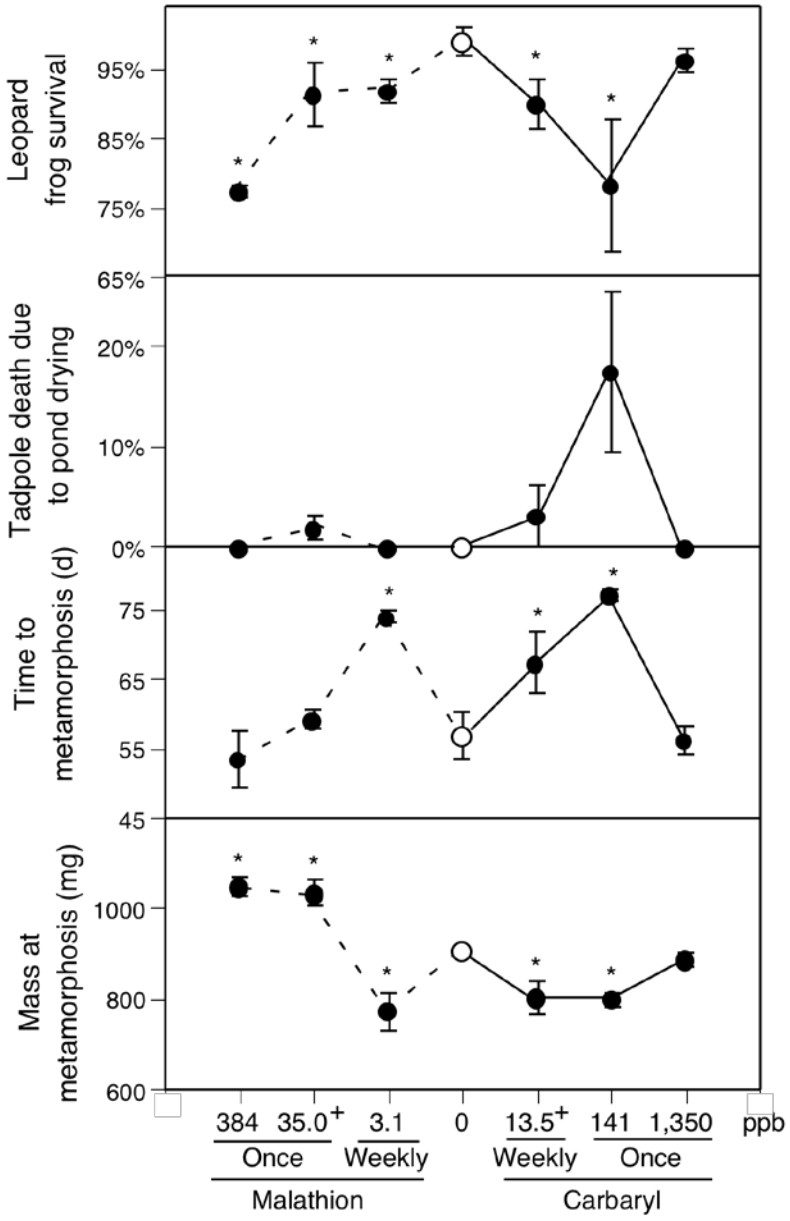


Figure 2.2. The effects of pesticide concentration, type (carbaryl or malathion), and timing of application (delivered weekly or once) on survival of leopard frogs *(Rana pipiens)*, proportion of leopard frog tadpoles that did not metamorphose before simulated pond drying as a result of slow development, time to metamorphosis and mass at metamorphosis. Data are means ± SE. Asterisks indicate treatments that are significantly different from the no-pesticide treatment ($P < 0.05$ using Fisher's LSD test). $^{+}$Actual concentrations were not available, values were estimated.

2.5 DISCUSSION

This experiment is one of only a few empirical tests examining the hypothesis that community-level of exposure to insecticides with similar modes of action and toxicity should result in comparable community level responses. While several studies have compared the effects of exposure to similar insecticides on zooplankton and phytoplankton, few have examined the wider range of community responses that were included in the current study (Van den Brink et al. 2002, Fleeger et al. 2003, Traas et al. 2004, Relyea and Hoverman 2006, Rohr et al. 2006, Clements and Rohr 2009). We compared the effects of two commonly used insecticides, carbaryl and malathion, at low weekly applications, single medium applications and single high applications to determine the concentrations at which community responses would be similar or divergent. In all insecticide treatments except the weekly low carbaryl treatment, insecticide exposure was lethal to some zooplankton and, in all but the weekly low and single medium carbaryl treatments, this death caused a trophic cascade that led to a bloom in phytoplankton. In some cases, the pesticides also reduced periphyton biomass. Tadpole life history was affected in all malathion treatments and the weekly low and single medium carbaryl treatments, however, the likely mechanism for these effects varied with the insecticide and concentration. Strong trophic cascades and early death of tadpoles affected the growth and development of tadpoles in the malathion treatments. In contrast, weak trophic cascades and strong direct effects of carbaryl on development appeared to cause slower development and growth in the two lower carbaryl concentrations, while tadpoles were unaffected in the high concentration. Thus, while both insecticides had similar effects on zooplankton and algae, they had different effects on amphibian survival and life history. As a result, limited generalizations about the community effects of these insecticides can be made at any application.

Both insecticides altered cladoceran populations. Exposure to malathion caused decreases in cladocerans, though this took longer to manifest in the weekly low treatment. Exposure to carbaryl caused decreases in cladocerans. The weekly low treatment recovered from these decreases by day 21, while the single medium and single high treatments did not. Previous research has found direct lethal effects of malathion on cladocerans at concentrations as low as 5 ppb in lab studies (Wong et al. 1995) and 10 ppb in mesocosm studies (Relyea and Diecks 2008). It appears that toxic effects in our weekly low malathion treatments (3 ppb each week for four weeks) took longer to manifest. Direct lethal effects of carbaryl on cladocerans have been recorded for concentrations as low as 15 ppb for *Daphnia sp.* in laboratory studies (Takahashi and Hanazato 2007) and 20 ppb for *Daphnia sp.* in mesocosm studies (Havens 1995). The concentration of carbaryl in our weekly low treatment (estimated at 13.5 ppb each week) appears to have been too low to have direct toxic effects on cladocerans. The observed increase in cladocerans in this treatment was driven primarily by a single replicate, which had more than four times more cladocerans than the average of the other three replicates for this treatment. This replicate did not qualify as a statistical outlier.

Copepods were also affected by some of the insecticide treatments. At weekly low and single medium exposures to both pesticides, whenever the abundance of cladocerans decreased, the abundance of copepods increased. In all cases, increases in copepods were not evident until day 21. This pattern has been observed in other insecticide studies and has been attributed to the lower sensitivity of copepods to insecticides combined with a competitive release of copepods caused by the decrease in cladocerans, which overlap with copepods in their consumption of phytoplankton (Hanazato and Yasuno 1987, 1989, Havens 1995, Mills and Semlitsch 2004, Relyea 2005b, Relyea and Diecks 2008). Compositional shifts of zooplankton assemblages are a

common response to insecticides including diazinon (Giddings et al. 1996), azadirachtin (Kreutzweiser et al. 2004), endosulfan (Barry and Logan 1998, Rohr and Crumrine 2005), esfenvalerate (Fairchild et al. 1992, Lozano et al. 1992) pyridaben, carbaryl (Hanazato and Yasuno 1987, 1989) and malathion (Relyea 2005a). To our knowledge, there are no studies exploring mechanisms underlying the high sensitivity of cladocerans to insecticides relative to the lower sensitivity of copepods.

The highest concentrations of each pesticide used in this experiment are known to cause copepod mortality (Relyea and Diecks 2008). Indeed, Hanazato and Yasuno (1989) observed direct toxic effects of carbaryl on all species of zooplankton at concentrations above 1000 ppb. This mortality may have offset the benefit of competitive release, resulting in no significant change in copepod abundance at the high concentrations of carbaryl and malathion.

Since zooplankton are the main consumers of phytoplankton in this system, we would expect decreases in zooplankton grazers to cause phytoplankton to bloom if phytoplankton growth is limited by herbivory. Chl *a* concentration increased three to nine times more than the control by day 21 in all malathion treatments, suggesting that herbivory did limit phytoplankton growth prior to the trophic cascade. Similar results were observed for the single high carbaryl treatment. The weekly low and single medium carbaryl treatments, which caused no declines or weak declines in cladocerans by day 21 respectively, caused no increase in phytoplankton. Phytoplankton blooms associated with insecticide applications have been previously documented with both organophosphate (Hurlbert 1972, Relyea and Diecks 2008) and carbamate insecticides (Mills and Semlitsch 2004).

Phytoplankton blooms are generally expected to reduce the amount of light that can pass through the water column; however, our measures of light extinction were not always associated

with phytoplankton. We found that the greatest light extinction coefficients occurred in the weekly low malathion treatment and the single high carbaryl treatment, which is consistent with the increases in phytoplankton observed in these treatments. The single medium and single high malathion treatments, which also had increases in chl *a*, did not have a significant increase in light extinction, though there was a non-significant trend. The sampling depth for light extinction was only 0.5 m, which may be too small for accurate measurements. Light extinction may also have been affected by factors other than phytoplankton, such as dissolved organic matter.

If the periphyton living on the bottom of a pond is light limited, shading from a phytoplankton bloom should decrease periphyton abundance. On the other hand, if periphyton growth is limited by herbivory from grazing tadpoles, increased mortality of tadpoles should increase periphyton as a result of decreased grazing pressure and from the input of nutrients from decomposing tadpoles. For malathion, after 21 d there was a small (but non-significant) reduction in periphyton with weekly low applications and a large reduction with the single medium application after 21 d, demonstrating a consistent negative effect of phytoplankton shading. Interestingly, the high malathion treatment displayed a rapid increase in periphyton early in the experiment (day 7). This is inconsistent with the time period (several weeks) that a trophic cascade would require to occur to affect periphyton abundance (Relyea and Diecks 2008, Relyea and Hoverman 2006). Instead, the increase in periphyton in the single high malathion treatment was likely caused by the death of tadpoles, which would have reduced periphyton consumption. Additionally, the high concentration of malathion may have favored an increase in periphyton by reducing tadpole foraging. Reduced foraging in response to AchE inhibition has been reported in similar studies (Bridges 1999, Weis et al. 2001).

For carbaryl, only the high single treatment, which caused a phytoplankton bloom and an

increase in light extinction, caused a reduction in periphyton. The reduction in periphyton with high carbaryl was likely caused by a trophic cascade that was initiated with the death of the cladocerans because these effects were only observed after 3 wks and tadpole survival was quite high (>95%), While a number of studies have observed pesticide-induced trophic cascades that affect zooplankton and phytoplankton (e.g., Boone et al. 2005, Boone and Bridges-Britton 2006), few studies have examined direct and indirect effects of pesticides on phytoplankton and periphyton (Mills and Semlitsch 2004, Relyea and Diecks 2008). This is an important oversight as these algal groups serve different functions in creating pond structure, cycling nutrients and as a resource for primary consumers.

The expected effect of pesticides on amphibians depends upon the direct toxic effects that could alter amphibian health and survival as well as the timing, extent and severity of trophic cascades that affect tadpoles' resources. Malathion reduced the survival of leopard frog tadpoles in all treatments even though the concentrations of malathion that tadpoles were exposed to (3, 35 and 384 ppb) were 1 to 3 orders of magnitude lower than LC50 values determined in lab environments (2400 ppb; Relyea 2004b). The U.S. EPA typically estimates the level of concern for pesticides at 5 or 10% of the LC50 value (Jones et al. 2004). Based on this reasoning, we would expect little or no tadpole mortality below 120 to 240 ppb (i.e. 5 to 10% of the leopard frog LC50). This was not the case.

Carbaryl decreased tadpole survival in the weekly low and single medium treatments. Unlike the mortality caused by malathion, mortality from carbaryl resulted from slower development and occurred because tadpoles could not metamorphose before their 'ponds' dried. This occurred at less than 10% of the predicted LC50 value for leopard frogs (LC50 = 2200 ppb; Relyea 2003c).

When resources are limiting, decreased tadpole survival should reduce competition. This can result in increased mass at metamorphosis and more rapid development of the survivors. Conversely, when a trophic cascade lowers periphyton abundance, tadpole competition should reduce growth and development. These traits are particularly important to amphibians. Larger mass at metamorphosis is associated with higher juvenile survival, improved mating success, earlier time to reproduction and production of higher quality eggs (Howard and Kluge 1985, Smith 1987, Altwegg and Reyer 2003). Earlier time to metamorphosis is associated with early reproductive success, increased survival and increased juvenile growth rate (Smith 1987, Altwegg and Reyer 2003).

Malathion exposure not only altered the survival of the leopard frogs, but also affected their development and growth. Leopard frogs receiving weekly low applications of malathion had reduced survival, emerged 3 weeks later and were 15% smaller than control frogs. In contrast, leopard frogs exposed to single medium or single high applications also had lower survival, but they emerged at the same time as the control and with larger mass. If the mortality occurred early in the experiment before the malathion broke down, which is the most likely scenario, this suggests that the reduced density of leopard frogs (and possibly reduced foraging activity of survivors) allowed a short-term increase in periphyton that was subsequently consumed by the remaining survivors and converted into an increased mass at metamorphosis. It is unclear why tadpoles in the weekly low treatment did not experience a similar increase in growth given that they also experienced a small decline in survival; a possible explanation would be if tadpole mortality occurred later in this treatment, leaving less time for survivors to benefit from reduced densities.

Among the carbaryl treatments, survival, development and growth were only affected by the weekly low and single medium treatments. Similarly to the weekly low malathion treatment, tadpoles in the these treatments had reduced survival, took longer to metamorphose and emerged at a smaller size. Neither of these treatments had reduced periphyton relative to the control on day 7 or 21. This suggests either that resources were limiting after day 21 so that high competition for resources resulted in reduced growth or that sublethal direct effects of insecticides may have reduced acquisition of resources or allocation towards growth and development. The latter explanation is more probable for the weekly low carbaryl treatment as there is little evidence for a trophic cascade at any point. Unlike the malathion treatments, tadpoles in these treatments did not experience reduced competition for periphyton as a result of tadpole mortality; most of the mortality occurred at the end of the larval period.

The single high carbaryl treatment had no effect on amphibian life history relative to the control. In this treatment, a trophic cascade beginning with a decrease in cladocerans led to a large decrease in periphyton on day 21; however amphibians had similar survival, mass and development time as the control despite a decrease in resources. This result is unexpected as tadpoles exposed to 10% as much carbaryl (in the medium treatment) experienced reduced survival, took on average 20 d longer to metamorphose and emerged at smaller sizes than the control. Similar patterns of decreasing effects of carbaryl on amphibian survival and life history traits with exposure to increasing concentrations have been found in some lab and mesocosm studies. For example, mesocosm studies of southern leopard frogs (*Rana sphenocephala*) show increasing survival and mass with increasing concentrations of carbaryl (3.5 and 7.0 ppm: Boone and James 2003; 2 and 5 ppm: Mills and Semlitsch 2004). Mesocosm studies of effects of carbaryl on American toads (*Bufo americanus*) and gray treefrogs (*Hyla versicolor*) across this

same gradient (3.5 and 7.0 ppm) show declining survival and increasing mass (Boone and Semlitsch 2001), while overwintered green frogs (*Rana clamitans*) in the same experiment were not affected by exposure to carbaryl. Boone and Semlitsch (2002) attribute these differences to the point in ontogeny in which organisms are exposed as well as the length of the larval period, with individuals with shorter larval periods being more negatively affected by exposure, however there are limited data to investigate this hypothesis.

Collectively, these results suggest that both insecticides can cause comparable trophic cascades that affect zooplankton and phytoplankton abundances; however their effects on amphibians diverge especially with exposure to higher concentrations of insecticides. While nearly all treatments caused trophic cascades initiated by decreases in zooplankton, exposure to a single application of more than 10% of the LC50 value for leopard frogs of carbaryl or malathion produced very different effects on amphibians and periphyton. We suggest that this is because malathion had early-acting direct lethal and sublethal effects on amphibians, while carbaryl had delayed sublethal effects on amphibians or no effects at all. Malathion caused reduced foraging and early death of tadpoles, while carbaryl exposure reduced growth, development and ultimately survival. Community responses may be similar for many contaminants when the main effect is driven through one causal pathway and the time frame of the effects is similar. When multiple direct and indirect trait- and density-mediated effects of pesticides on communities interact, the community response depends on the strength and timing of these interactions and is less generalizable (e.g. Relyea and Hoverman 2006). To develop predictive models for the effects of contaminants on communities we need to incorporate sublethal (as well as lethal) effects of contaminants into community ecology based models and focus on the time-frame of these effects. Traditional toxicity tests, which focus on mortality and not behavioral and

developmental effects may not provide sufficient information for the development of such models.

3.0 PREDATOR-CUES, BUT NOT PESTICIDES, REDUCE SKIN PEPTIDES YET IMPROVE SURVIVAL AGAINST PATHOGENIC FUNGI (*BATRACHOCHYTRIUM DENDROBATIDIS*) IN POST-METAMORPHIC WOOD FROGS (*RANA SYLVATICA*)

3.1 ABSTRACT

Batrachochytrium dendrobatidis (Bd), the fungal pathogen that causes chytridiomycosis in amphibians, is found on six continents and is contributing to amphibian population declines. It is hypothesized that natural and anthropogenic environmental factors may contribute to this trend by reducing the immunocompetence of pathogen hosts, making them more susceptible to infection. Antimicrobial peptides (AMPs) produced in the granular glands of a frog's skin are thought to be a key defense against Bd infection. These peptides may be a critical immune defense at metamorphosis because other acquired immune functions are suppressed during this transition. To test if stressors altered AMP production and disease susceptibility during this life history transition, we exposed wood frog (*Rana sylvatica*) tadpoles to the presence or absence of dragonfly predator cues crossed with a single exposure to three nominal concentrations of the insecticide malathion (0, 10 or 100 ppb). We then exposed a subset of the post-metamorphic frogs to the presence or absence of Bd zoospores and measured frog survival. While predator cues and malathion had no effect on metamorph survival or growth, predator cues increased the time to metamorphosis by 1.5 d and decreased the amount of hydrophobic skin peptides by 20%.

Despite this decrease in peptides, previous exposure to predator cues increased survival in both Bd-exposed and unexposed frogs, suggesting that the sublethal stress of predators can confer a fitness advantage. Overall, these results suggest that tadpole exposure to predator cues reduces the amount of skin peptides released onto the skin and can confer fitness benefits later in life regardless of whether Bd is present or absent.

3.2 INTRODUCTION

Emerging infectious diseases (EIDs) are increasing rapidly in number and severity on a global scale (Daszak et al. 2000, Jones et al. 2008). Altered environmental conditions are thought to contribute to these patterns (Jones et al. 2008, Hayes et al. 2010, Martin et al. 2010). For example, chytridiomycosis in amphibians, which is caused by infection with the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), has been identified as an EID that is contributing to amphibian population declines around the world (reviewed in Skerratt et al. 2007, Fisher et al. 2009, Kilpatrick et al. 2010). Bd is found on six continents in over 350 species and appears to be the proximate cause for extinction for some of these species (Fisher et al. 2009). At the same time, environmental changes, including habitat degradation, exposure to contaminants, and increased UV-B exposure may also contribute to amphibian population declines (reviewed in Beebee and Griffiths 2005). It is hypothesized that these stressors may interact with Bd to make amphibians more vulnerable to infection (Carey et al. 1999, Beebee and Griffiths 2005, Hayes et al. 2010, Blaustein et al. 2011); however we know very little about what mechanisms or stressors could be driving these interactions.

Several hypotheses have recently been proposed that relate increased prevalence and virulence of disease with environmental factors (Lafferty 1997, Carey et al. 1999, Rollins-Smith 2001, Blaustein and Kiesecker 2002, Martin et al. 2010, Marcogliese and Pietrock 2011). In this study we focus on the hypothesis that susceptibility to disease increases because exposure to stressors reduces the immunocompetence of the host (Carey et al. 1999). This could occur directly if the stressor inhibits components of the innate or acquired immune system, or indirectly, as a result of neuroendocrine-immune crosstalk or reduced allocation of resources towards immune functions (Carey et al. 1999, Martin et al. 2010). We know that many anthropogenic stressors, especially contaminants, can be immunosuppressive for amphibians (e.g. Kiesecker 2002, Christin et al. 2003, Gendron et al. 2003, Forson and Storfer 2006, Hayes 2006, Rohr et al. 2008a, b carbaryl: Davidson et al. 2007a) but little is known about how natural stressors alter amphibian immunocompetence (Gervasi and Foufopolous 2008) or if multiple stressors have synergistic impacts on immunocompetence.

Correlational evidence supports the idea that environmental variation may influence the epidemiology of Bd. Within species, population declines in Bd-affected areas are often correlated with other abiotic co-factors (e.g., moisture, temperature, altitude, UV-radiation, Retallick et al. 2004, Ron 2005, Pounds et al. 2006, Bielby et al. 2008, Ortiz-Santaliestra et al. 2011). In particular, insecticides are thought increase susceptibility to infection (e.g. Carey et al. 1999, Rollins-Smith and Conlon 2005, Hayes et al. 2010, Rollins-Smith et al. 2011). Upwind applications of carbamate and organophosphate insecticides that share a mode of action (acetylcholine esterase (AchE) inhibition) have been correlated with amphibian declines (Davidson et al. 2001, 2002, Davidson 2004). AchE activity in Pacific tree frogs (*Pseudacris regilla*) is also reduced at sites where co-occurring species are declining (Sparling et al. 2001).

However, the pesticides in these ponds are found at concentrations that are too low to be directly lethal to amphibians, suggesting that these pesticides may affect amphibians through mechanisms other than direct toxicity, such as immunosuppression. Little is known about how biotic co-factors may influence amphibians exposed to Bd (e.g. Parris et al. 2004, 2006, Han et al. 2011).

Among the hundreds of pesticide active ingredients on the market (Jones et al. 2004, Giagnessi and Reiger 2006a, b), malathion may be especially relevant for immunsuppression of amphibians. First, malathion is an organophosphate insecticide that acts by irreversibly binding to AchE. Moreover, in the United States, malathion is the most commonly used insecticide, with 9 to 11.3 million kg of active ingredient used annually in agriculture and additional millions of kg used in gardening and public health programs (Kiely et al. 2001). Finally, while malathion is moderately lethal to amphibians with LC50 values for larval anurans of 1.3 to 5.9 mg/L (Relyea 2004b, USEPA Ecotox database, *available online* http://cfpub.epa.gov/ecotox/), it may have strong sublethal effects (e. g. Groner and Relyea 2011). Organophosphorous chemicals such as malathion are associated with immunotoxic effects in vertebrates (reviewed in Galloway and Handy 2003). These effects can occur directly, if the contaminant impairs immune functions, or indirectly through modulation of the nervous system or by limiting energy available for developing and maintaining the immune system as a result of lowered metabolism or malnutrition. Several studies show that malathion suppresses cellular and humoral immune components of amphibians (Kiesecker 2002, Gilbertson et al. 2003, Budishak et al. 1999) or increases prevalence of infection in frogs (e.g., lungworms, trematodes and *Aeromonas* bacteria; Taylor et al. 1999, Kiesecker 2002, Christin et al. 2003, Gilbertson et al. 2003); however, no studies have examined the effects of malathion exposure on innate immune functions.

Immune responses to Bd are not thoroughly understood, though evidence shows that both innate and acquired immune functions may be important (Rollins-Smith 2001, Rollins-Smith and Conlon 2005, Richmond et al. 2009, Ramsey et al. 2010, Voyles et al. 2010, Rollins-Smith et al. 2011). Some of the best evidence for an immune defense against Bd comes from studies of the suite of antimicrobial peptides (AMPs) released onto the surface skin (reviewed in Rollins-Smith and Conlon 2005, Ramsey 2010, Rollins-Smith et al. 2011). Antimicrobial peptides are stored in granular glands beneath the skin and released onto the skin when norepinephrine induces the contraction of the smooth muscles these glands. Because these peptides break down quickly, peptides are thought to only be present on the skin for a brief period following peptide release (reviewed in Rollins-Smith et al. 2005). AMP production is relatively slow; frogs depleted of AMPs can take more than 30 days to replenish granular glands (Ramsey et al. 2010). Many AMPs inhibit growth of Bd *in vitro* (Rollins-Smith et al. 2002a, b, c, 2005, reviewed in Rollins-Smith and Conlon 2005, Rollins-Smith 2009, Ramsey et al. 2010) and production of inhibitory AMPs is correlated with resistance across species (Woodhams et al. 2006, 2007). Moreover, AMPs are hypothesized to influence the composition of beneficial bacteria on the skin, which has been shown to inhibit Bd growth *in vitro* and *in vivo* (Harris et al. 2006, 2009, reviewed in Rollins-Smith 2009, Rollins-Smith et al. 2011).

The role of AMPs may be heightened during metamorphosis. During this time many components of the acquired immune system (e.g., lymphocyte numbers and viability, mitogen-induced proliferation) are suppressed to prevent autoimmunity against new adult-specific antigens (Rollins-Smith et al. 1997, reviewed in Rollins-Smith 1998); however, AMPs are first released in substantial quantities at metamorphosis when the granular glands mature (Bovbjerg 1963). In many species, most of the mortality due to infection occurs during or soon after

metamorphosis (Bosch et al. 2001, Briggs et al. 2005, Bosch and Martínez 2006, Garner et al. 2009). Moreover, for at least one species (*R. luteiventris*), infection loads in sampled populations are the highest, repeatedly, at this stage of development (Russell et al. 2010). Thus, AMPs and the skin bacteria that they influence may play an important role in defense against infection during this critical time (Woodhams et al. 2006, Harris et al. 2006).

Environmental stressors including contaminants, predators, and harmful microbes may alter AMP synthesis and release onto the skin (Rollins-Smith and Conlon 2005, Rollins-Smith et al. 2011). For example, production of AMPs is inhibited by release of glucocorticoids, which are often elevated during responses to environmental stressors (Simmaco et al. 1997, Romero 2004). Therefore increased production or altered sensitivity to this stress hormone would be predicted to alter AMP production. Effects of stressors on AMPs can also depend on the type of stress experienced. For example, exposure to predators, as well as pursuit by simulated predators, induces the release of peptides in frogs and, in at least one case, peptide release was shown to predation by stimulating oral dyskinesia (e.g., yawning) in the predator (Barthalmus and Zielinski 1988, Ramsey et al. 2010, reviewed in Daly et al. 1987, Bevins and Zasloff 1990). Therefore, chronic exposure to predators might be expected to deplete peptide stores or lead to compensatory increases in AMP production. Other stressors are shown to reduce the concentration of AMP releases. For example, carbaryl (an AchE inhibiting insectide) can reduce AMP releases in young metamorphs or adults (Davidson et al 2007, Schadich et al. 2009). Despite compelling immunological and epidemiological arguments, no studies have examined how exposure of larval amphibians to contaminants or other stressors affects AMP production at metamorphosis.

In this study, we tested the general hypothesis that natural and anthropogenical stressors induce immunosuppression and lead to increased disease susceptibility in amphibians. We exposed wood frogs (*Rana sylvatica*) to two different stressors—predators and pesticide exposure—and subsequently examined susceptibility to Bd in a subset of animals exposed to these stressor treatments. We predicted that: 1) exposing tadpoles to malathion will suppress skin peptide production in metamorphs; 2) exposing tadpoles to predator cues will suppress skin peptide production in metamorphs; 3) the effects of predators and malathion on skin peptide production of the frogs will be non-additive; and 4) decreased production or release of skin peptides will be associated with increased susceptibility to chytridiomycosis in postmetamorphic frogs exposed to Bd.

3.3 METHODS

3.3.1 Experimental Design

The study was conducted in two stages. The first stage tested the effects of exposing wood frog tadpoles to predators and pesticides on the life history traits and releases of AMPs onto the skin of newly metamorphosed wood frogs (metamorphs). The second stage used these metamorphosed wood frogs to assess the effects of larval exposure to these stressors on the susceptibility of metamorphs to Bd. The first stage was conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology in Linesville, Pennsylvania, USA; the second stage was conducted at Oregon State University in Corvallis, Oregon, USA.

Tadpole exposure to natural and anthropogenic stressors was accomplished using a completely randomized full-factorial design containing three malathion treatments (nominal concentrations of 0, 10 and 100 ppb) crossed with two predator treatments (predator cues present or absent). The 0 ppb malathion treatments were replicated 6 times and all other treatments were replicated 5 times. Experimental units were plastic wading pools (mesocosms) filled with 100-L of well water on 5 April 2009. On 16 April we added to these mesocosms, 5 g of rabbit chow to serve as a nutrient source, 100 g of leaf litter to serve as a substrate for periphyton, and aliquots of pond water from three local ponds to establish populations of periphyton and phytoplankton. On 21 April we added zooplankton collected from two local ponds. Thus, the wading pools contained several components of natural wetlands. Wood frogs from 26 egg masses were collected from three ponds in Crawford County between 20 March and 2 April and raised in predator-free culture pools. All culture pools and mesocosms were covered with mesh lids to exclude colonization by predators or escape of metamorphs. On 8 May, hereafter 'experiment day 1' we added 20 wood frog tadpoles to each mesocosm (Gosner stage 25; Gosner 1960, mean mass ± SE = 78 ± 4 mg). Twenty-four hour survival of these animals after handling was 100%.

The predator treatments consisted of exposure to the presence or absence of predator cues. Our predators were locally collected larval dragonflies (*Anax junius*). This species has a cosmopolitan distribution throughout North America and are common predators of wood frogs (Corbet 1999). *A. junius* were caged in 200-mL plastic cups with a mesh screen and fed 298 ± 3 mg (mean ± SE) of wood frog tadpoles three times per week. Tadpoles respond to cues from digested conspecifics, so caged predators can induce a response without attacking focal animals (Schoeppner and Relyea 2009). The concentration of cue in this experiment (cues from 3 mg digested tadpoles / L of water) is sufficient to induce the maximum plastic response in wood

frogs (Schoeppner and Relyea 2008). Mesocosms assigned the no-predator-cue treatment contained empty cages. On feeding days, these empty cages were lifted out of the water and put back to equalize the disturbance across all mesocosms. Predator treatments began on day 4 and continued until day 32. Predators that died or stopped feeding were replaced with similarly sized *Anax junius*.

Because we were interested in how exposure of tadpoles to pesticides affected immune function in metamorphs, we applied pesticide treatments on day 28, which we estimated to be 1 wk before the onset of metamorphosis, based on the developmental stage of tadpoles at that point. The formulation of malathion (Gordon's® malathion, 50% active ingredient) was diluted to a stock solution of 18.9 µg/L and we added 0, 51.8 or 518 µL of this stock solution to the appropriate mesocosms to achieve nominal concentrations of 0, 10 and 100 ppb. These concentrations are within the range that have been detected in natural ponds (Odenkirchen and Wente 2007). Pesticides were stirred into each mesocosm with a plastic cup. Two hours after the application, water samples were collected and pooled within pesticide treatments for confirmation of nominal concentrations. Samples were stored at 4 °C and then shipped for analysis by high-pressure liquid chromatography (Mississippi State Chemical Laboratory, Mississippi State, MS).

Analyses of the water samples indicated that the actual concentrations of pesticides were 0.24, 2.8 and 32 ppb for the 0, 10 and 100 ppb nominal concentration treatments, respectively (Mississippi State Chemical Laboratory, Mississippi State, MS). While the actual concentrations deviated from the nominal concentrations, the deviation was a consistent 68-72% reduction from the targeted concentrations. Variation from nominal concentrations is frequently found in mesocosm experiments (reviewed in Brock et al. 2000) and is thought to arise from precipitation

or volatization of pesticides in mesocosms, incomplete mixing of pesticides within mesocosms as a result of binding to substrates (Farmer et al. 1995), or degradation of stored samples before the assay (Crum and Brock 1994, Farmer et al 1995, Brock et al. 2000, van Wijngaarden et al. 1996). The source of trace levels of malathion in the control is unknown.

3.3.2 Response Variables

To test the effects of treatments on water quality, we measured dissolved oxygen (DO), temperature, and pH between 1300 and 1500 hrs on experiment day 32. Temperature and DO were measured with a calibrated MultiLine P4 meter (Wissenschaftlich-Technische Werkstatten, Weilheim, Germany) and pH was measured with a calibrated pH meter (testr 10 double junction, Oakton, Vernon Hills, USA).

The first tadpoles began to metamorphose on day 33. We placed two pieces of 30-cm long wood strips into all mesocosms on day 32 to provide a perch for metamorphs to prevent drowning. After day 33, mesocosms were checked for metamorphs daily. Animals with emerged forelimbs were removed from mesocosms and housed in 1-L containers with sphagnum moss until they resorbed their tails to < 2mm. At this stage of tail resorption metamorphosis was considered complete and we weighed the metamorph. After weighing, eight individuals from each treatment were isolated for peptide induction and 12 individuals from each treatment were set aside for a future challenge with Bd. Metamorphs used for AMP collection were held individually in 590-mL plastic cups containing sphagnum moss and fed crickets *ad libitum*. Metamorphs used for the Bd challenge were grouped by mesocosm in 14-L tubs containing sphagnum moss and fed crickets dusted with ReptoCal™ *ad libitum*. Once all metamorphs had emerged, the animals were overnight-shipped to Oregon State University.

3.3.2.1 Antimicrobial Peptide Collection

Hydrophobic skin peptides were measured after metamorphosis was completed. We measured hydrophobic skin peptides from, on average, 6.2 frogs per replicate, and 31 frogs per treatment. Since we did not measure skin peptides from all frogs, we chose a subset of frogs from each replicate that equally represented frogs that metamorphosed early, midway and late in the experiment (the 2^{nd}, 5^{th}, 7^{th}, 10^{th}, 13^{th}, 15^{th}, 17^{th} and 20^{th} frog to emerge). For each frog, we waited 8 d after the completion of metamorphosis before extracting AMPs to allow animals to acclimate to their new environment. These animals were weighed right before AMP extraction.

AMPs were extracted with a subcutaneously injection of 2 nmol norepinephrine-HCl / g frog. This injection triggers contraction of the smooth muscle surrounding the granular glands that store AMPs, causing them to be released (reviewed in Rollins-Smith and Conlon 2005). Therefore measurements of AMPs reflect sensitivity to norepinephrine as well as AMP storage at the time of release. After injection, frogs were placed into 10 mL of collection buffer (25 mM ammonium acetate and 25 mM NaCl, pH 7.0, Rollins-Smith et al. 2002a). After 10 min, frogs were returned to plastic cups and the collection buffer was acidified with 500 µL of trifluoracetic acid and frozen. The concentration of norephinephrine used in this experiment is thought to be comparable to levels experienced during stressful events in nature. For example, frogs chased by a researcher's hand released the same amount of peptides as frogs injected with 2 nmol Norepinephrine-HCl/ g frog and significantly more peptides than resting frogs (Ramsey et al. 2010).

After collection, hydrophobic peptides were partially purified from this buffer and enriched by passage over C-18 sep-pak cartridges (Goraya et al. 1998, Goraya et al. 2000,

Rollins-Smith et al. 2002a). Peptides were then eluted in 70% acetonitrile, 29.9% water and 0.1% trifluoracetic acid. Peptide concentrations were determined using the Micro BCA analysis (Pierce, Rockford, IL) following the kit instructions, with the exception that bradykinin was used to establish a standard curve (Smith et al. 1985).

3.3.2.2 Antimicrobial Peptide Characterization

Only one peptide has been sequenced for wood frogs, and there are no studies of peptides released by wood frog metamorphs (Mattute et al. 2000). Since most ranids produce a diverse suite of AMPs (Rollins-Smith 2005), we expected to find novel peptides in our samples. Determination of peptide sequences was done using nano-flow electro-spray liquid chromatography quadrapole tandem mass spectrometry (Q-TOF II ESI/APCI Quadrupole-TOF, Waters Corporation). Samples from each treatment were combined and loaded onto an 100 μ (ID) column containing ~10 cm of C-18 packing. Mass spectra were first obtained in the 550-3000 m/z range and promising spectra (with charges >1) were targeted for MS/MS. Assignment of sequences was done manually using the methods described in Kinter and Sherman (2000).

3.3.2.3 Stage 2: Bd Challenge

At Oregon State University, animals were placed in glass terraria (grouped by mesocosm) in a laboratory maintained at a temperature of 21.5 to 23.3ºC with a 13:11 light:dark photoperiod. All animals were given 1 wk to acclimate to these conditions before being weighed and moved to individual experimental enclosures (plastic Petri dishes, 140 mm diameter x 30 mm height, with air holes in the lids). Petri dishes contained 10 ml of dechlorinated water and 15 ml of Bd

inoculate or sham inoculate. Animals were able to partially climb the walls of the Petri dish, but could not completely lift themselves off the bottom, keeping them in constant contact with the water. Animals were kept in these dishes for the duration of the experiment and fed three pinhead crickets twice a week (Searle et al. 2011).

3.3.2.4 Bd culturing and inoculation

We infected metamorphs with Bd strain JEL 258, originally isolated from a wood frog in Maine. Bd was cultured on 1% tryptone agar plates that were made 15 d prior to inoculation and held at approximately 22ºC. To harvest Bd from agar, we flooded plates with 15 mL dechlorinated water for 5 min to allow for zoospores to be discharged from sporangia. To standardize inoculation dose among Petri dishes, all flooded Bd plates were scraped to remove zoospores and this suspension was pooled to create a single inoculation broth. Zoospore concentration in the inoculation broth was determined by hemocytometer and diluted to a standard working concentration of ~6.3 x 10^3 zoospores per mL. Control animals were given a similar treatment except sterile agar plates were flooded with dechlorinated water. All experimental animals were exposed to the same Bd concentration (~9.45 * 10^4 zoospores/ plate) at each inoculation. Two experimental inoculations took place over the course of the infection experiment, on day 1 (July 14) and day 9. Inoculations were not initiated until 2 d after metamorphs were moved into their Petri dishes, so that they could acclimate to their new environments. Water in Petri dishes was changed during reinoculation. Throughout this stage of the study, animals (n = 245) were checked twice daily for mortality. Animals that died during the experiment were removed from their dishes and preserved in 95% ethanol. The experiment was terminated upon the death of the

last animal in the Bd treatment (day 16) and all remaining control animals were humanely euthanized by immersion in MS-222.

To determine if experimental infection was successful and to test for cross-contamination, several animals from each treatment were preserved in 95% ethanol and the infection status of toeclips of 10 animals from Bd treatments and 10 from Bd controls was determined using quantitative PCR. We followed the protocol outlined in Boyle et al. (2004) with the exception that toe clips were macerated with a mortar and pestle instead of a bead beater. All samples were run in triplicate. All 10 Bd exposed individuals tested positive for infection and none of the 10 control animals tested positive for infection. The mean infection load for Bd exposed frogs was 8400 ± 2100 zoospores.

3.3.3 Statistical Analyses

For the first stage of the experiment, we tested the effects of malathion and predator exposure on water quality, life history traits of wood frogs, and AMP production in newly metamorphosed wood frogs. For the second stage of the experiment, we tested the effects of Bd exposure, malathion exposure, and predator exposure on the survival of postmetamorphic wood frogs.

We tested the effects of malathion and predator-cues on water quality variables (pH, dissolved oxygen, temperature) using multivariate analysis of variance (MANOVA). All water quality variables met assumptions of equal variances and normality. Because we found no effects of the treatments on water quality variables, we will not discuss them.

We tested the effects of the malathion and predator-cues on metamorph response variables (size at metamorphosis, time to metamorphosis and survival to metamorphosis) using MANOVA. Whenever a multivariate effect was significant, we conducted ANOVAs on each

response variable. The proportion surviving was arcsine square-root transformed to meet the assumptions of normality. These data still did not meet the assumption of equal variances, because the variation in the 10 ppb malathion*predator-cue treatment was smaller than other treatments. We used Pillai's trace to test for significance, as it is robust to minor violations of this assumption (Quinn and Keough 2002, p. 434).

We used a nested ANCOVA to test the effects of treatments on AMP production. Since bigger animals need more peptides to protect a greater skin surface area, we used mass as a covariate. To control for the non-independence of metamorphs that were reared in the same mesocosm, we included mesocosm as a random nested term within the predator-by-pesticide interaction. Total skin peptide concentrations were log-transformed to meet the assumption of normality. No assumptions were violated in this test.

To test how malathion, predator-cues and Bd affected post-metamorphosis survival, we used a Cox's proportional hazards model (Cox 1972). This method allowed us to determine how experimental treatments altered the probability of death relative to the probability of death in a control treatment (e.g., the hazard ratio). Since a proportional hazard model uses individual data and individuals that shared a mesocosm as tadpoles may have correlated responses, we initially included mesocosm as a block nested within treatment. This term was not significant in our model (χ^2=26.1, p = 0.4591), so we present the model without it. Our initial model also showed no significant treatment interactions, so we excluded interactions from the final model. Proportional hazards models assume that covariates multiply the hazard experienced relative to a baseline risk at a constant rate over time. For our data, the Bd treatment violated the assumption of proportional hazards because the risk increased dramatically in the Bd treatments several days after the experiment began, presumably because the infection had increased to a critical level at

this time. To meet the proportionality assumption, we ran the model stratifying Bd into two time-dependent levels. The results of this test were the same as the initial model, so we just present the former. The proportional hazards model was run using SAS (v. 9.2) and all other statistical analyses were run using SPSS (v. 18).

3.4 RESULTS

3.4.1 Amphibian growth, development and survival to metamorphosis

The analysis of wood frog life history traits detected a multivariate effect of predator cue (Pillai's Trace $F_{3, 24} = 3.964\ 8$, $P = 0.020$) but not pesticide (Pillai's Trace $F_{6,50} = 1.028\ 8$, $P = 0.418$) or the predator-pesticide interaction (Pillai's Trace, $F_{6,50} = 0.665\ 8$, $P = 0.678$; Figure 1). Subsequent univariate analyses indicated that the multivariate predator-cue effect was driven by an effect of predators on time to metamorphosis ($F = 9.987$, $P = 0.004$), but not on survival ($F = 1.519$, $P = 0.229$) or mass at metamorphosis ($F = 1.962$, $P = 0.173$). Individuals exposed to predator cues took an average 1.5 d longer to metamorphose than individuals not exposed to predator cues (Figure 3.1).

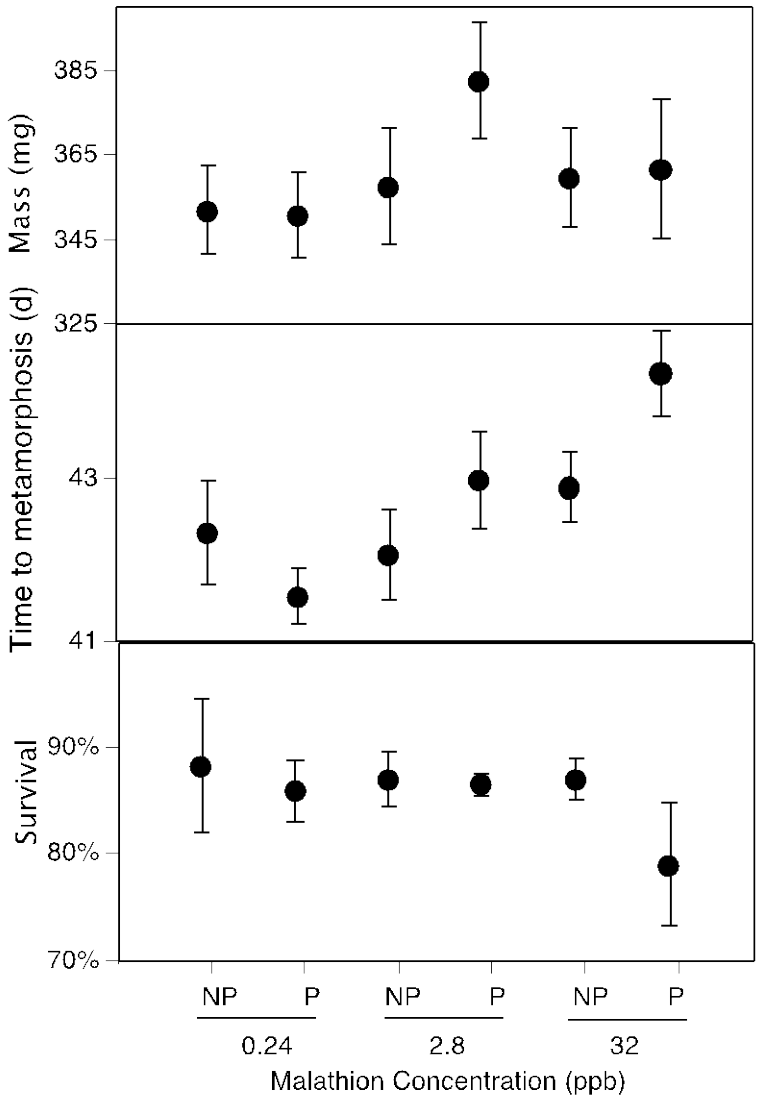


Figure 3.1. Effects (means ± SE) of malathion concentration (0.24, 2.8, or 0.32 ppb) and predator treatment (predator cues = P, no predator cues = NP) on mass of wood frogs at metamorphosis, time to metamorphosis, and survival to metamorphosis. Exposure to predator cues caused tadpoles to metamorphose ~1.5 days later than animals exposed to no-predator cues. No other effects were significant.

3.4.2 Release of skin peptides

Averaged across all malathion treatments, skin peptides were 20% lower for animals that were exposed to predators compared to those not exposed to predators (Table 3.1, Figure 3.2). This effect was marginally significant. While there was not a significant malathion-by-predator interaction, this effect was stronger in the 0 and 10 ppb treatments. There was also a marginally significant effect of the predator-by-mass interaction and a significant effect of replicate on skin peptides. To better understand the predator-by-mass interaction, we examined the effect of the mass covariate within each predator-cue treatment. In the no-predator treatments, skin peptides increased by 1.75 ug for every 1 mg increase in mass. This explained about 3% of the variation in peptide production in this treatment ($r^2 = 0.031$). In contrast, there was no correlation between peptide production and mass in the predator treatment ($r^2 = 0.0007$). In short, mass explained very little of the variation in peptides measured within each predator treatment. Malathion exposure had no effect on the production of antimicrobial peptides.

Table 3.1. Results of a partially nested ANCOVA testing the effects of exposure of wood frog tadpoles to three malathion concentrations (0.24, 2.8 or 32 ppb) and two predator treatments (predator cues or no predator cues) on the production of skin peptides 1 wk after metamorphosis. In this analysis mass was used as a covariate and the mesocosm where tadpoles were raised (replicate) was nested as a random effect within fixed treatment effects. Both predator and not predator 0.24 ppb malathion treatments were replicated 6 times. All other treatments were replicated five times. Bold fonts indicate p-values that are < 0.1.

Effects	**F (df)**	***p***	***Partial Eta Squared***
Mass	0.980 (1, 157)	0.324	0.006
Predator	**3.679 (1, 26)**	**0.097**	**0.102**
Pesticide	1.234 (2, 26)	0.308	0.087
Replicate (Predator * Pesticide)	**1.675 (26, 157)**	**0.029**	**0.217**
Predator * Pesticide	0.179 (2, 26)	0.837	0.014
Pesticide * Mass	1.253 (2, 26)	0.302	0.88
Mass * Predator	**4.633 (1, 26)**	**0.065**	**0.125**
Mass * Predator * Pesticide	0.157 (2, 26)	0.882	0.10

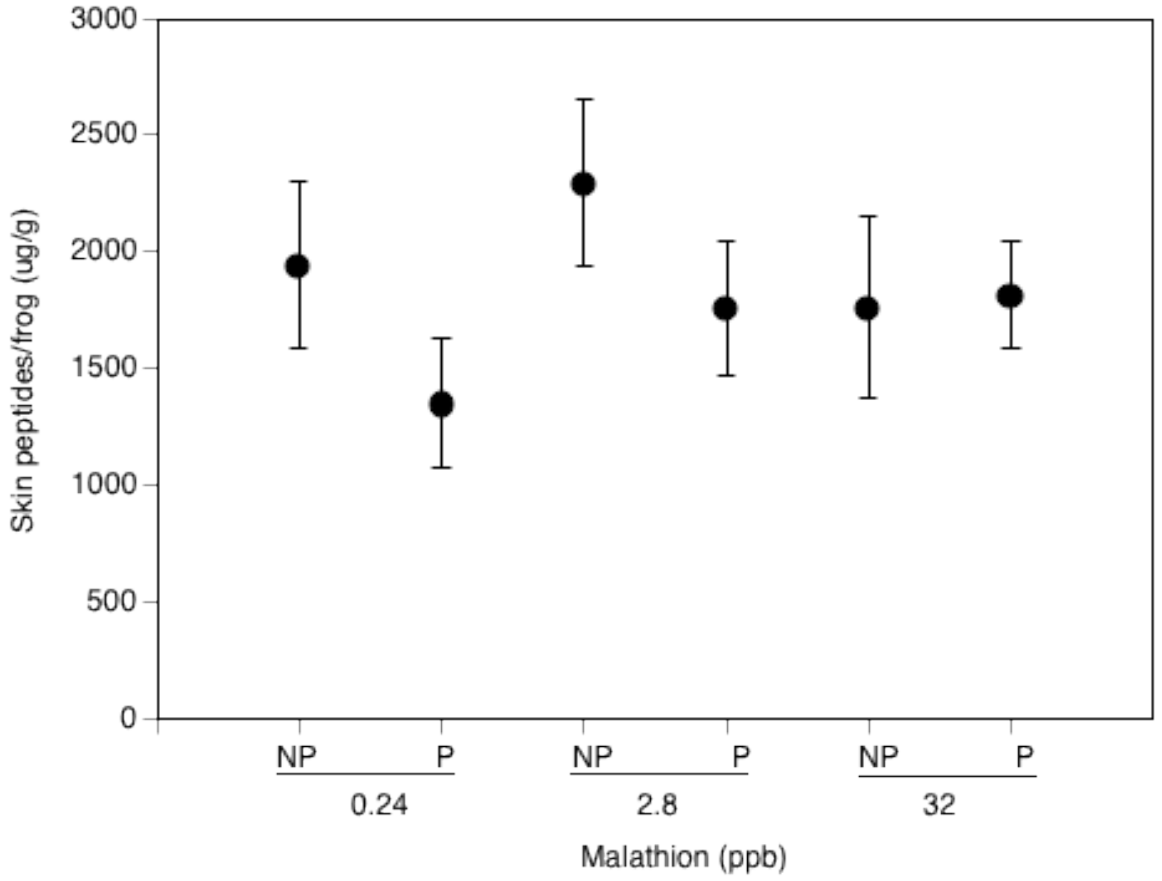


Figure 3.2. Effects (means ± SE) of malathion concentration (0.24, 2.8, or 0.32 ppb) and predator treatment (predator cues = P, no predator cues = NP) on mass-adjusted production of peptides in the skin of wood frogs. To obtain the peptides, frogs were injected with 2 nmol norepinephrine-HCl/ g eight days after metamorphosis. A nested ANCOVA showed that prior exposure to predators caused a 20% decrease in total skin peptides. There was no effect of malathion or malathion-by-predator cue interaction.

3.4.3 Antimicrobial peptide characterization

Using mass spectrometry, we detected signals of five peptides in our samples. Of these, full amino acid sequences could be determined for two of them, based on spectra from tandem mass spectrometry (Table A1.1). One of them matched the sequence for brevinin-1SY, which has been previously described in wood frogs by Mattute et al. (2000). While this peptide has been observed in adult wood frogs, this is the first time it has been observed in recently metamorphosed wood frogs. The other peptide we have designated temporin-1SY according to a widely accepted convention for naming new peptides (Conlon 2008). This peptide yielded a sequence that has not been previously characterized and which we believe to be a temporin (Figure A1.1). The y1 series of this peptide was compatible with an 18 dalton increase over the residue mass, suggesting that the C-terminal residue was amidated, as is characteristic of temporin peptides. Moreover, it has a 27% match with a temporin consensus sequence and 100% homology in terms of its hydrophobicity and hydrophilicity (Wade 2010). The closest match to temporin-1SY is temporin-PRa, which had 50% similarity and is produced by the Oregon spotted frog (*R. pretiosa*; Conlon et al. 2011; see Table A1.1 for similar temporins).

3.4.4 Bd challenge

There was a significant effect of Bd across all treatments (Figure 3.3, Table 3.2). Exposure to Bd increased the risk of mortality nearly 8-fold. This hazard was reduced in the predator treatment. Exposure to predator cues decreased the risk of mortality in both Bd-exposed and unexposed treatments by nearly 50%. Pesticide exposure and treatment interactions had no effect on the proportional hazards function.

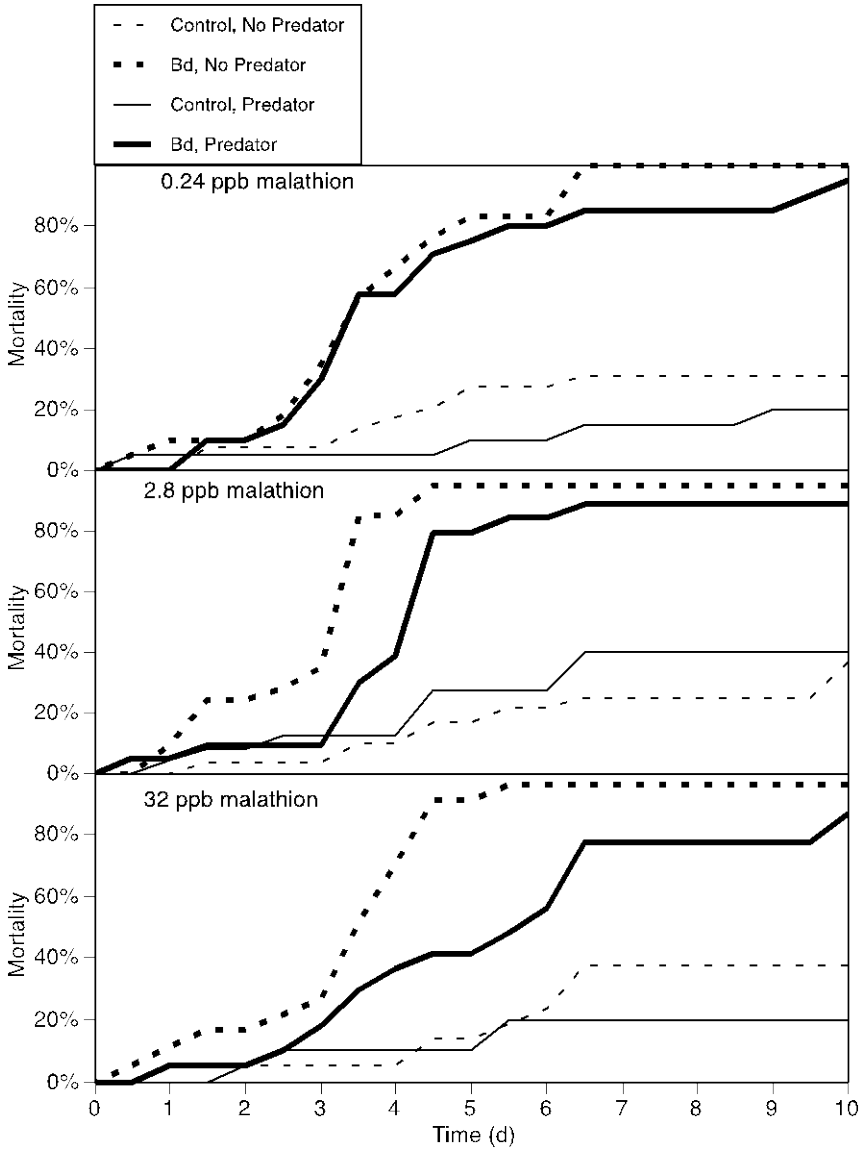


Figure 3.3. Effects of exposure of wood frog tadpoles(*Rana sylvatica*) to malathion (0.24, 2.8, or 0.32 ppb) and caged predators or no predators on the survival of metamorphic wood frogs when exposed to infectious zoospores of the fungal pathogen, *Batrachochytrium dendrobatidis* or a control broth lacking zoospores (n = 245). A Cox's proportional hazards model showed that exposure to *Batrachochytrium dendrobatidis* significantly increased the risk of death by a factor of 8, while exposure to predators decreased the risk of death by nearly 50%.

Table 3.2. Effects of exposure of wood frog tadpoles to three malathion concentrations (0.24, 2.8, or 32 ppb), two predator treatments (predator cues or no predator cues) and the fungal pathogen Bd on the risk of metamorph mortality. Results are from a Cox's proportional hazard. For each variable chi-squared values, degrees of freedom and *p*-values are presented. Hazard ratios and 95% confidence limits are included where relevant. Low malathion indicates a pairwise comparison of the low malathion treatment (2.8 ppb nominal concentration) to the control, while high malathion indicates a pairwise comparison of the high malathion treatment (32 ppb) to the control. Bold font indicates p -values less than 0.05.

Model	Chi-squared (df)	p-value	Hazard ratio	Lower 95% Confidence Limit	Upper 95% Confidence limit
Main effects	**130.8 (4)**	**<0.001**			
Bd	**107.7 (1)**	**< 0.001**	**7.915**	**5.355**	**11.699**
Predator cues	**9.5 (1)**	**0.002**	**0.595**	**0.438**	**0.827**
Pesticide	2.5 (2)	0.284			
Low malathion	0.5 (1)	0.458	1.159	0.785	1.712
High malathion	0.7 (1)	0.390	0.839	0.563	1.251

3.5 DISCUSSION

This study tested the general hypothesis that predator cues and pesticides can alter the immunity and survival of animals exposed to pathogens. It has been proposed that natural and anthropogenic stressors can lead to lead to increased infection prevalence, disease susceptibility and disease-induced population declines because they depress immune functions (Carey et al. 1999, Rollins-Smith 2001, Blaustein and Kiesecker 2002, Hayes 2010, Martin et al. 2010). Past tests of this hypothesis often just focused on immunocompetence or disease susceptibility, but not both (reviewed in Graham et al. 2011). Our study focused on both response variables. We examined how stressors experienced during the larval stage affected immune function and diseases susceptibility in newly metamorphosed frogs which have a limited immune capacity and often experience high rates of disease mortality in the field (Rollins-Smith 1998, Kiesecker 2002, Gilbertson et al. 2003, Briggs et al. 2005). We found that predator cues decreased immune responses, yet improved post-metamorphic survival. Specifically, we found a (marginally significant) reduction in the amount of hydrophobic skin peptides in wood frogs exposed to predator cues. Moreover, predator cues increased survival of both Bd-exposed and unexposed frogs. There was no effect of short-term malathion exposure (beginning about 5 d prior to the beginning of metamorphosis) on the production of skin peptides quantified 8 d after metamorphosis or on susceptibility to Bd. To our knowledge, this study is the first study of the effects of any natural stressor on the production of skin peptides in frogs.

3.5.1 Immunocompetence

Exposure to predator cues caused a (marginally significant) decrease in the amount of hydrophobic peptides released after a mild stimulus with norepinephrine. While we did not quantify concentrations of specific antimicrobial peptides in different treatments, the 20% reduction in total skin peptides caused by predators could potentially decrease concentrations of AMPs below those that inhibit infection (Rollins-Smith 2002a). In this experiment the sample sizes for AMP measurements from each treatment were larger than those typically used in studies of AMP releases (e.g., Davidson et al. 2007, Tennessen et al. 2009, Ramsey et al. 2010). However, due to the high variation in this trait, the power of this analysis was still low.

While chapter 4 of this thesis found that variation in life history traits could explain effects of stressors (predator-cues and competition) on AMPs, this explanation does not extend to this study. Consistent with previous studies, predator cues caused minimal changes in life history patterns. Tadpoles exposed to predator cues took ~1.5 d longer to metamorphose but did not differ in growth or survival relative to control animals. Because we standardized the measurements of AMPs to control for time since metamorphosis, we do not think that this relatively small decrease in developmental rate explains the effect of predator cues on skin peptides.

Several physiological mechanisms could explain the effect of predator cues on skin peptides. Skin peptide measurements are a function of peptide synthesis rates, time since the last peptide release and sensitivity to stressors which trigger the norepinephrine induction of peptide release from granular glands. Since antimicrobial peptide releases are triggered by predators (Ramsey et al. 2010), exposure to predator cues during development may have caused more frequent peptides releases, which could have led to depleted peptide stores in the granular glands

at the time of measurement. Predator cues were administered until day 32 and some tadpoles began metamorphosis at day 33. This means that peptides were measured frogs could restore depleted peptide stores (estimated to be 30-40 d, Ramsey et al. 2010). In addition, predator cues may have caused tadpoles to reduce allocation to peptide production or interfered with peptide production near the time of metamorphosis, thereby reducing the amount of peptides available for release (Ramsey et al. 2010). Finally, exposure to chronic stresses such as predators could cause altered sensitivity to stress hormones such as norepinephrine and glucocorticoids, which are involved in triggering and inhibiting AMP releases (McEwen and Seeman 2006). Further studies of predator effects on AMP production near the time of metamorphosis would assist in parsing apart these explanations.

We hypothesized that exposure to malathion would also reduce skin peptide defenses in wood frogs because malathion alters neuroendocrine signaling which is necessary to stimulate release of peptides (Rollins-Smith et al. 2005). This would be especially likely if exposure to malathion leads to a sympathetic stress response, which could result in increased episodes of peptide release and temporary peptide depletion (Rollins-Smith 2001). We did not find an effect of malathion on the production of skin peptides or survival of recently metamorphosed frogs exposed to Bd. The lack of a malathion effect may have been due to low concentrations used or due to the short duration of exposure.

Previous studies exploring effects of contaminants on AMPs (Davidson et al. 2007, Schadich et al. 2009) found that exposure to much higher concentrations (~480 ppb) of carbaryl (also an AchE-inhibiting insecticide) reduced AMPs collected from post-metamorphic frogs (*R. boylii* and *Litoria raniformis*) 2 to 3 d after exposure. The timing of exposure also differed; Davidson et al. (2007) exposed metamorphs to the insecticide whereas we exposed late-stage

tadpoles. Short-term immunosuppression of amphibians may not have as much biological relevance for explaining patterns of Bd infection in the wild unless populations are repeatedly immunosuppressed (e.g., by repeated exposure to these pesticides).

In the current study, malathion treatments were applied in a manner that would isolate direct effects of malathion on immune functions. Thus, applications were at a much lower concentration than what is lethal to amphibians (e.g.,LC50 values range from 1.3 to 5.9 mg/L, Relyea 2004b) and they were applied late in development (~1 wk before metamorphosis), so that amphibians metamorphosed before trophically-mediated indirect effects of exposure could affect life history traits and resource acquisition. As we expected, this late, brief application had no effect on survival, growth or development. However, exposing tadpole to malathion early enough in their ontogeny to alter growth and development could indirectly lead to altered AMP production by altering acquisition and allocation of resources to life history traits and immune function (Relyea and Diecks 2008, Groner and Relyea 2011).

The AMPs observed by mass spectrometry in this study have the potential to inhibit Bd. Although the potency of brevinin-1SRY against Bd has not been tested, other brevinins (from other ranid frogs) have varying degrees of antimicrobial activities against pathogens, including Bd (reviewed in Rollins-Smith and Conlon 2005, Rollins-Smith 2009). The presence of this peptide in metamorphs suggests that they may be able to use AMPs to defend themselves against microbial parasites during this vulnerable life stage. Temporins can also inhibit Bd growth *in vitro* (Rollins-Smith et al. 2003). The level of inhibition is dependent upon the ability to attach to the fungal cell membrane, the formation of α-helix after attachment (thought to be necessary for membrane disruption) and resistance against fungal proteases (Rollins-Smith et al. 2003). Temporin-1SY is predicted to form α-helix, which would yield seven hydrophobic amino acids

on a single surface, indicating that it has strong membrane binding potential (Wang and Wang 2004). Molecular analyses of synonymous mutations of peptide gene sequences suggest that both temporins and brevinins have been under diversifying selection across populations and balancing selection within populations (Duda et al. 2002, Tennessen and Blouin 2007). Moreover, regional variation in peptides produced by *R. pipiens* suggests that peptides are adapted to respond to local microbial threats (Tennessen et al. 2009). Collectively, this evidence suggests that the peptides identified in this study may have been selected for by the local microbial environment.

3.5.2 Disease Susceptibility

We hypothesized that a decrease in the amount of skin peptides would be correlated with increased susceptibility to Bd. Contrary to our expectation, we found that animals exposed to predator cues had 60% higher survival in both the Bd and control treatments. Acclimation to stress can result from early exposure to high amounts of stress, which results in reduced stress responses later in life (Dobrakova et al 1993, Caldji et al. 2001, Martin 2009). Additionally, chronic exposure to stressors can alter stress responses as a result of increased physiological costs of frequent stress activation (e.g., allostatic load, reviewed in Romero 2004, McEwen and Seeman 2006). It is possible that the metamorph environments in the second stage of the experiment (i.e. Petri dishes) were stressful and that frogs that had previously experienced the stress of predators were better adjusted able to withstand these environments. Understanding the mechanism and conditionality behind beneficial responses to stresses earlier in life will aid in interpreting how early and chronic exposure to stressors and allostatic loads manifest later in life.

Exposure to malathion did not alter susceptibility to Bd. Although malathion did not alter skin peptide production, other symptoms of malathion exposure could have caused decreased

survival in Bd-exposed animals. For example, AchE inhibition causes reduced swimming speed and activity in tadpoles (Bridges 1997). If it has the same effect on metamorphs, deleterious effects of reduced acquisition of resources resulting from decreased activity could lead to increased morbidity or mortality. Mortality from infection occurred so rapidly in this study that we did not test for effects of exposure on infection loads, growth or behavior. Thus, we cannot rule out the possibility that malathion could alter responses to less virulent strains of Bd.

3.5.3 Synthesis

The unexpected relationship between skin peptide production and susceptibility to Bd demonstrates the complex nature of the relation between immune function and disease. We predicted that decreases in skin peptides would lead to an increase in disease mortality. However, we found that newly metamorphosed frogs that had been exposed to predators during their time as tadpoles produced fewer skin peptides and survived longer in the presence and absence of Bd. These results suggest that acclimation in tadpoles exposed to predators conferred a fitness benefit during the disease challenge. Additionally, the amount of peptides produced in these animals were may not have been within the range or of the right type that they were effective against such concentrated exposures to this strain of Bd that was so virulent to wood frogs. More studies that test effects of stressors on both immune function and disease will elucidate mechanisms underlying this complex relationship and help to develop more predictive models.

Across the literature, the wide range of responses of amphibians to predator and contaminant stressors suggests a need for further refining of the environmental immunosuppression hypothesis. For example, sublethal predator exposure did not alter immune functions (leukocyte counts or types) or infection rates of trematodes (*Echinostomata trivolvis*)

in American toad tadpoles (*Bufo americanus*; Raffel et al. 2010). In contrast, Navarro et al. (2004) found decreased T-cell mediated immune response and higher malarial loads in house sparrows exposed to predators. Additionally recent hypotheses have suggested that instead of stress immunosuppressing individuals, it may lead to a redistribution of resources towards various immune efforts (the immunoredistribution hypothesis, Braude et al. 1999, Martin et al. 2006). Some of this variation in responses may be explained by length of the exposure to a stressor, the developmental stage of the organism, the specific immune function tested and the life history of the pathogen. Inclusion of this variation in the environmental immunosuppression hypothesis may yield more useful predictions about effects of stressors across life stages.

3.6 CONCLUSIONS

Understanding how multiple stressors interact to affect survival and physiology is an important goal of ecology. This is an especially timely challenge in the case of EIDs, where there is a pressing need to understand how natural and anthropogenic stressors in the environment alter immune function and disease rates. Recent hypotheses suggest that environmental stressors negatively affect immune function and disease susceptibility of amphibians (Carey et al. 1993, 1999, Rollins-Smith 2001). This experiment found that exposure of wood frog tadpoles to predator cues tended to reduce the production of skin peptides in newly emerged metamorphs. However, this reduction was not associated with increased disease susceptibility in predator-exposed animals. Instead, predator stress increased survival of both Bd and control animals. Positive effects of predator stress on animals later in life suggests that stresses experienced early in development prime individuals to have resist stress later in life. We did not find any effects of

exposure to malathion on production of skin peptides or disease susceptibility. In contrast, other experiments testing effects of malathion exposure on cellular and humoral immune responses in adult amphibians have found strong negative effects; however they have not examined delayed effects of stress exposure across life history stages. We encourage more exploration to reveal the mechanisms underlying variation in effects of stressors on immune functions, disease susceptibility and acclimation to future stress.

4.0 EFFECTS OF PREDATOR CUES ON INNATE IMMUNE FUNCTIONS OF RECENTLY METAMORPHOSED LEOPARD FROGS (*RANA PIPIENS*) ARE MEDIATED BY THE COMPETITIVE ENVIRONMENT AND THE LENGTH OF EXPOSURE

4.1 ABSTRACT

Amphibians are experiencing rapid population declines across the globe due, in part, to emerging infectious diseases. Environmental immunosuppression may be contributing to these trends by diminishing resistance to infectious diseases; however, there are few tests of this hypothesis. Moreover, those that exist are often conducted under unrealistic lab conditions and focus on acquired, but not innate immune functions. Antimicrobial peptides (AMPs) in the skin of amphibians are an important innate immune defense against fungal, viral and bacterial pathogens and their release is tightly coupled with release of the stress hormone, norepinephrine. During metamorphosis, AMPs are one of the only functioning immune responses because acquired immune functions are temporarily suppressed in order to prevent autoimmunity against new adult antigens. Suppression of AMPs during this transitional stage may impact disease rates.

We exposed Northern leopard frog tadpoles (*Rana pipiens*) to a factorial combination of competitive and caged-predator environments and measured their development and growth as well as the amount of hydrophobic peptides in the skin after metamorphosis. Exposure to

predators altered the production of skin peptides, but the effect depended upon the level of competition. While metamorphs exposed to no predator cues increased peptide releases under high competition, metamorphs exposed to chronic predator cues or early predator cues had little or no change in peptide releases under high competition. Many of these patterns can be explained by the variation in timing of metamorphosis relative to exposure to predator-cues as well as possible plasticity in the rates of AMP synthesis in response to chronic stress. Characterization of collected peptides from these frogs show that they include previously uncharacterized brevinins and temporins, which have previously been shown to inhibit growth of Batrachochytrium dendrobatidis, the fungal pathogen associated with global amphibian declines.

4.2 INTRODUCTION

Emerging infectious diseases are increasing rapidly around the world, both in number and severity. While the causes for these trends are poorly understood, changing environmental conditions are thought to alter the epidemiology of many pathogens (Jones et al. 2008). Recent papers have focused on the effects of environmental stress on immune responses and how that might impact disease incidence (Råberg et al. 1998, Carey et al. 1999, Hawley and Altizer 2010). For example, amphibians are declining around the globe and emerging infectious diseases are contributing to these trends (Daszak et al. 2003, Collins and Storfer 2003). Recent literature suggests stress-induced immunosuppression may play a role in amphibian declines, by increasing infection prevalence or infection loads—but the hypothesis requires a great deal of research to understand when and how it may apply (Carey 1993, Carey et al. 1999, Rollins-Smith 2001, Daszak et al. 2003, Rachowicz et al. 2005, Fisher et al. 2009). We are just beginning to

understand patterns of natural variation in immune functions vary in nature and the role of stressors in immunomodulation (reviewed in Martin 2009, Martin et al. 2011).

Many of the pathogens that negatively impact amphibian populations can infect the skin. These include the bacteria *Aeromonas hydrophila*, the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), the water mould *Saprolegnia ferax and iridoviruses* (Hird et al. 1981, Longcore et al. 1999, Rollins-Smith 2001, Romansic et al. 2009). However, amphibians have a suite of innate and acquired defenses against skin infection (Clarke 1997, Richmond et al. 2009, Rollins-Smith et al. 2011). One important innate immune response against skin-infecting pathogens is the array of antimicrobial peptides (AMPs) that are released onto the skin as part of the sympathetic stress response. AMPs from frogs have been found to inhibit the growth of gram-negative and gram-positive bacteria and fungal pathogens including Bd, the fungal pathogen that causes chytridiomycosis in amphibians (Conlon et al. 2004, Rollins-Smith and Conlon 2005, Rollins-Smith et al. 2002b, c). In addition to killing some pathogens, it is hypothesized that AMPs also influence the diversity of beneficial skin bacteria that inhibit the growth of detrimental pathogens (Harris et al. 2009).

It is hypothesized that stress may alter the amount of AMPs released onto the skin (reviewed in Rollins-Smith et al. 2011). AMPs that are the most potent against Bd are found in species that appear to be less impacted by Bd infections (Woodhams et al. 2006); however, some populations of frogs that produce AMPs that inhibit Bd *in vitro* still suffer from chytridiomycosis and experience disease-related population declines (e. g., Briggs et al. 2005, Tobler and Schmidt 2010). It is unknown how much of this variation is due to environmental stressors that alter the immunocompetence of exposed amphibians (Gervasi and Foufopolous 2008, Fisher et al. 2009, Richmond et al. 2009, Rollins-Smith et al. 2011).

The stress of predation and competition for resources may have particular relevance for the production and release of AMPs. Exposure to predators, as well as pursuit by simulated predators, induces the release of peptides (Barthalmus and Zielinski 1988, Bevins and Zasloff 1990, Clarke 1997, Ramsey et al. 2010, chapter 3). In addition to altering releases of AMPs, exposure to predators alters many plastic traits including life-history responses and inducible defenses (e.g. Relyea 2001, 2003, Van Buskirk 2009). The magnitude of these responses often decrease in highly competitive environments as a result of reduced acquisition of resources, altered allocation of resources, and altered neuroendocrine signaling (e.g., Rollins-Smith 1998, Relyea 2004a). Thus, we suspect that competition may also alter, predator- or pathogen-induced releases of AMPs, though this has never been tested.

Though it is rarely studied in nature, the timing and duration of exposure to these stressors may also influence immune functions (Apanius 1998, Martin 2009). In particular, chronic exposure to stressors can cause both hypo- and hyper-responsiveness to stressors later in life, presumably as a result of altered release of stress agonists or activity of receptors to stress hormones (Romero 2004, Denver 2009, Martin 2009). Chronic activation of the 'stress axis' (hypothalamic-pituitary-interrenal axis) is also hypothesized to cause more frequent peptide releases, potentially depleting AMP stores over time (Rollins-Smith 2001, Ramsey et al. 2010, Rollins-Smith et al. 2011). The timing of exposure to environmental stressors may influence AMPs by altering the sensitivity of the stress axis later in life (Romero 2004, Denver 2009, Martin 2009). Exposure to stressors early in development results in increased neuroendocrine stress axis activity later in life (Hu et al. 2008, Martin 2009). Moreover, tadpoles injected with stress hormones (e.g. corticosterone) show altered neuroendocrine gene expression later in life (Denver 2009). Later in ontogeny the stress axis may be more robust to stressors (Martin 2009).

Collectively, these data suggest that chronic or early exposure to stressors could alter the production of stress hormones, which are related to the production and release of AMPs. To our knowledge, effects of the timing and length of stressor exposure on AMPs have not been tested.

The role of AMPs is heightened during amphibian metamorphosis (Rollins-Smith 2001, 2009). Many components of the acquired immune system (lymphocyte numbers and viability, and mitogen-induced proliferation) are inhibited during metamorphosis in order to prevent autoimmunity against the new adult-specific antigens produced during this period (Rollins-Smith 1998, Bosch et al. 2001, Briggs et al. 2005, Bosch and Martínez 2006, Garner et al. 2009). AMPs begin to be released in substantial quantities during metamorphosis, although the suite of peptides synthesized may only be a subset of the full adult set (Bovbjerg 1963, Woodhams and Rollins-Smith, unpublished). Substantial mortality and morbidity due to disease often coincide with metamorphosis, however it is unclear whether this is a result of reduced immune function, increased exposure to pathogens or some combination (e.g., Green et al. 2002, Bosch and Martínez 2006, Garner et al. 2009). Only three studies have characterized AMPs produced at the time of metamorphosis and explored how stressors affect AMP concentrations released onto the skin (Davidson et al. 2007, Schadich et al. 2009, chapter 3).

We explored how the timing and duration of predator stress under low and high competition during the larval stage affected the amount of skin peptides released onto the skin of recently metamorphosed leopard frogs (*Rana pipiens*, metamorphs). In doing so, we tested four hypotheses: 1) the stress of competitors and predators would reduce AMP production, 2) chronic stress would reduce AMP production more than acute stress, 3) acute stress early in development would reduce peptides more than acute stress late in development, and 4) predator and competitor stress would have synergistic effects on AMP production. To understand how AMPs

were influenced by the life history variation that these treatments induce, we also measured growth, development and survival of the frogs. We characterized AMPs that we collected using mass spectrometry in order to understand the function and identity of these peptides.

4.3 METHODS

4.3.1 Experimental Design

We conducted the experiment in the spring and summer of 2009 at the Pymatuning Laboratory of Ecology (Linesville, Pennsylvania, USA). We used a completely randomized design composed of factorial combination of four predator cue treatments and two competition treatments. The four predator cue treatments consisted of no predator cues, an early exposure to predator cues, a late exposure to predator cues or a chronic exposure to predator cues throughout the larval period. The two competition treatments consisted of low and high tadpole densities.

The eight treatments were replicated four times for a total of 32 experimental units. Each experimental unit consisted of an 800-L mesocosm filled with 600-L well water (pH = 8), 15 g of rabbit chow to serve as a nutrient source and 200 g of dry leaf litter (primarily *Quercus sp.*) to serve as a substrate for algal growth. On 16 April we also added pond water, collected from several local sites, to each mesocosm, to serve as a source of microbiota and plankton. On 21 April and 2 May, we added additional water collected from several local ponds to each mesocosm to further bolster microbial populations. Mesocosms were covered with shadecloth to prevent colonization by flying insects and escape of metamorphosing frogs.

We collected eight leopard frog egg masses from a local pond and allowed them to develop in outdoor mesocosms until they reached Gosner stage 25 (Gosner 1960, mean mass ± 1 SE = 32.0 ± 1.7 mg). At this point they were added to the experimental mesocosms (5 May). Unfortunately, some wood frog tadpoles (*R. sylvatica*) were accidentally mixed into the culture pools of leopard frog tadpoles. As a result, wood frogs comprised 8.6 ± 1.2 % of the individuals in each tank. However, statistical tests (described below) indicated that wood frog additions did not alter the results of the experiment.

The four predator treatments were designed to vary both the timing and duration of exposure to predator cues relative to tadpole ontogeny. The early-cue treatment was a 1-wk pulsed exposure to caged predators during week 1 of the experiment. The late-cue treatment was a 1-wk pulsed exposure to caged predators during week 5 of the experiment. The chronic-cue treatment was an exposure to caged predators until the tadpoles metamorphosed (6.5 weekss). The no-cue treatment was an exposure to empty predator cages. Hereafter, these treatments will be referred to as the early-cue, late-cue, chronic-cue, and no-cue treatments.

To create the predator cues, we collected dragonfly larvae (*Anax junius*) from local ponds. These dragonflies have a cosmopolitan distribution in the United States and commonly consume leopard frog tadpoles. Each mesocosm contained two 800-mL plastic drain pipes secured with mesh screens held in place with rubberbands. These cages were suspended at the water surface and *A. junius* were held in each container during the relevant exposure periods for each treatment. Caged *A. junius* were fed leopard frog tadpoles (300 ± 10 mg each) to the predators three times per week. Predators that were not feeding or died were replaced with new *A. junius* at the next feeding. The amount of cue produced from digested conspecifics was concentrated enough to induce plastic morphological, physiological and behavioral responses

(Schoeppner and Relyea 2008). The first predator feeding was on 11 May (defined as experiment day 1) and the last feeding was on day 45, just before leopard frog tadpoles began to metamorphose.

Intraspecific competition was manipulated by altering tadpole densities. Low competition treatments contained 15 tadpoles whereas high competition treatments contained 30 tadpoles. This range of densities (18-37 tadpoles/ m^3) is within observed densities of leopard frog tadpoles in the wild.

Wood frog tadpoles began to metamorphose on day 32 and leopard frogs began to metamorphose on day 46. After the first wood frog metamorphs appeared, tanks were checked daily. Individuals with 4 emerged limbs were collected and held in 1-L containers with sphagnum moss until metamorphosis was complete. We considered metamorphosis complete when the tail was resorbed to less than 2 mm. After metamorphosis was complete, individuals were weighed and placed in 600-mL cups with mesh lids containing wet sphagnum moss. Each juvenile leopard frog received two ~6 mm crickets that were dusted with the calcium supplement Reptocal™.

4.3.2 Skin Peptide Collection

We measured hydrophobic skin peptides on recently metamorphosed frogs because this life stage experiences significant disease-related mortality (e.g. Bosch et al. 2001, Green et al. 2002, Bosch and Martínez 2006, Garner et al. 2009). We attempted to collect skin peptides from eight frogs per replicate; however we averaged five frogs because, in some replicates not enough animals survived or metamorphosed before the experiment was ended. Since we did not measure skin

peptides from all frogs, we chose animals that represented early-, mid- and late- developers from each experimental unit.

We induced the release of antimicrobial peptides from the skin glands using norepinephrine-HCl dissolved in amphibian buffered PBS. Norepinephrine induces the contraction of the smooth muscles surrounding the granular glands, where AMPs are stored (Holmes and Balls 1978). Upon contraction, peptides are released onto the skin. Peptides are thought to only be present on the skin for the period immediately following peptide release because they break down rapidly (reviewed in Rollins-Smith and Conlon 2005). Frequent or large releases of peptides can deplete peptide storages for more than 30 days (Ramsey et al. 2011). Our measurements of AMP levels reflect sensitivity to norepinephrine, the rate of production of AMPs and the frequency of peptide releases.

We used 20 nmol /g frog of norepinephrine-Hcl to allow the release of a detectable amount of peptides without approaching a maximum level of release (Ramsey et al. 2010). Frogs chased by a researcher's hand released the same amount of peptides as frogs injected with 2 nmol norepinephrine-HCl/g frog and significantly more peptides than resting frogs (Ramsey et al. 2010). Maximum peptide release is achieved with an injection of 80 nmol norepinephrine/g frog. Past studies of antimicrobial peptides in metamorphs show that they have lower concentrations on their skin relative to adults (Woodhams and Rollins-Smith, unpublished), so we used more than might be released during a natural stress event so that we could extract a detectable amount of peptides. After injection with norepinephrine, the frogs were immediately immersed in 45 mL of collection buffer (25 mM ammonium acetate and 25 mM NaCl, pH 7.0) for 10 min. After this, we removed the frog and then acidified to peptides with 450 uL of trifloracetic acid and froze them at -20 °C (Rollins-Smith et al. 2002a). Hydrophobic peptides

were partially purified and collected onto C-18 sep-pak cartridges (Goraya et al. 1998, Goraya et al. 2000, Rollins-Smith et al. 2002a). Peptides were then eluted in 70% acetonitrile and concentrations were determined using Micro BCA analysis (Pierce, Rockford, IL) following the kit instructions, with the exception that bradykinin was used to establish a standard curve (Smith et al. 1985).

4.3.3 Antimicrobial Peptide Characterization

Initial attempts to determine peptide sequences were conducted using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). However, inhibition of desorption/ionization was suspected and confirmed when adding our samples to known standards prevented detection of a signal. This has been observed for AMPs collected from metamorphs on three separate instances and for two frog species, suggesting that the inhibiting compound is released by metamorphic frogs (chapter 3, unpublished data). Ultimately, determination of peptide sequences was done using nano-flow electro-spray liquid chromatography coupled with quadrapole time of flight tandem mass spectrometry (Q-TOF II ESI Quadrapole-TOF MS/MS, Waters corporation). Purified samples were concentrated using a lyophilizer, combined across treatments to increase the amount to detectable levels and loaded onto a column containing ~10 cm of C-18 packing (inner diameter 100 μ). Mass spectra were first obtained in the 550-3000 m/z range and promising spectra (with charges >1) were targeted for MS/MS. Manual assignment of sequences followed approaches described in Kinter and Sherman (2000).

4.3.4 Statistical analyses

We used a multivariate analysis of variance (MANOVA) to test the effects of competition and predator cues on metamorphosis response variables (size at metamorphosis, time to metamorphosis and survival to metamorphosis). We conducted ANOVAs on significant variables in the MANOVA. Pairwise comparisons were made with Tukey's HSD test.

Tadpole survival and mass at metamorphosis violated the assumption of sphericity (Box's $M_{42,\ 952.633} = 0.007$); however this assumption was met in MANOVAs that tested the effects of either competition or predator cues, suggesting that the violation was fairly minor. Because we were most interested in the results of ANOVAs and switching to a non-parametric test would obfuscate many of the trends in the MANOVA, we decided to proceed with the original test, despite this violation. We used Pillai's trace, which is more robust to violations of sphericity (Quinn and Keough 2002, pg. 434). To meet the assumption of normality, data on time to metamorphosis were square-root transformed and data on the proportion of tadpoles surviving to metamorphosis were arcsine square-root transformed.

To test if the accidental inclusion of wood frogs into treatments affected these response variables, we included the number of wood frogs that were accidentally added to each tank as a covariate. However, this term was not significant (Pillai's Trace $F_{3,\ 21} = 0.6$, $P = 0.981$), so we excluded it from the final model.

We used an ANOVA to test the effects of treatments on the tank means of mass-standardized antimicrobial peptides (ug AMP/ g of frog). Since bigger animals need more peptides to protect a greater skin surface area, this is a standard adjustment to make to total peptide production (e.g. Davidson et al. 2007). Due to the volume of peptide samples that we had to process, some samples were processed immediately after elution, while others were stored at -

80 °C and processed several days later. Peptide samples that had been frozen at -80 °C were statistically different from all other samples after controlling for treatment effects (using ANOVA). Since these samples were all substantially lower, we suspect that they had degraded as a result of freezing and thawing and we excluded these samples from the analysis. This reduced our sample size by 26%. Mass standardized antimicrobial peptides were log-transformed to meet the assumption of normality. Because we had *a priori* hypotheses about differences between predator treatments and interactions with competition, we did not adjust for multiple comparisons (Quinn and Keough 2002, pp. 49). No assumptions were violated in this test.

4.4 RESULTS

4.4.1 Life history traits

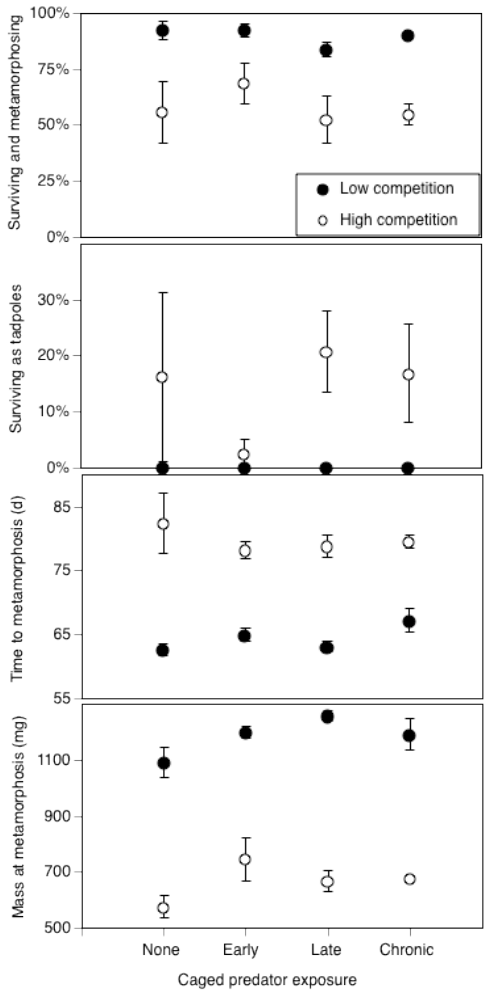


Figure 4.1. Effects of competition and predator cue treatments applied to leopard frog (*Rana pipiens*) tadpoles on their survival to metamorphosis, survival of tadpoles that did not metamorphose, development and growth. Data are means ± SE. Analysis of variance showed that higher densities of tadpoles significantly decreased survival, development and growth rates, while exposure to predators significantly increased mass at metamorphosis relative to animals not exposed to predators.

The MANOVA on survival to metamorphosis, time to metamorphosis and mass of leopard frogs at metamorphosis showed significant effects of competition (Pillai's Trace $F_{3, 21} = 92.7$, $P < 0.001$) and predator cues (Pillai's Trace $F_{9, 69} = 2.4$, $P = 0.022$), but not their interaction (Pillai's Trace $F_{9, 69} = 0.9$, $P = 0.494$).

The multivariate effect of competition was driven by all three response variables (Figure 4.1, Table 4.1). Compared to the low competition treatments, 35% fewer leopard frogs metamorphosed in the high competition treatments. Of those animals that did not metamorphose, 55% failed to metamorphose because they died during the experiment while 45% did not metamorphose because they did not develop rapidly enough before the experiment ended. Frogs in the high competition treatments also metamorphosed 15 d later and were 44% less massive than frogs in the low competition treatments.

The multivariate effect of predator cues was driven by mass at metamorphosis (Figure 4.1, Table 4.1). Compared to the no-cue treatment, frogs in the early-cue and late-cue treatments were 16% larger (Tukey's HSD, $p < 0.03$). The predator cue treatments had no effect on survival or time to metamorphosis. Frogs in the chronic-cue treatment were not different from the no-cue, early-cue or late-cue treatments (Tukey's HSD, all $p > 0.13$).

Table 4.1. Results of ANOVAs on the effects of competition and predator cues on the survival to metamorphosis, mass at metamorphosis and time to metamorphosis of leopard frogs. For each response variable, *F*-values are listed first followed by *P*-values in parentheses.

Effect	df	Survival to Metamorphosis	Mass at Metamorphosis	Time to Metamorphosis
Model	(7, 24)	6.391	41.263	18.078
		(0.001)	(0.001)	(0.001)
Competition	(1, 24)	39.6	274.2	120.7
		(0.001)	(0.001)	(0.001)
Predator cues	(3, 24)	1.317	4.082	0.627
		(0.290)	(0.018)	(0.590)
Competition* Predator cues	(3, 24)	0.388	0.806	1.35
		(0.763)	(0.503)	(0.282)

4.4.2 Skin peptides

There was a significant univariate effect of competition and a marginal competitor-by-predator interaction on mass-standardized skin peptides (Figure 4.2, Table 4.2). The marginal interaction occurred because the magnitude of peptide increases between low and high competition varied with predator treatment. In the no-cue treatment, high competition caused a 268% increase in mass-standardized peptide production (LSD, $p = 0.007$). The early-cue and late-cue treatments, competition caused 198% and 564% increases in mass-standardized peptide production, respectively (LSD, $p = 0.052$, $p < 0.001$, respectively). In the chronic-cue treatment, however, competition had no effect on skin peptides (LSD, $p = 0.219$).

We can also examine the effects of predator cues within each competition treatment. Under low competition, mean comparisons indicated that mass-standardized skin peptides were not different from each other (LSD, all $p > 0.16$) except for the late-cue treatment which was 61% lower than the chronic-cue treatment (LSD, $p = 0.04$). Under high competition, mean comparisons indicated that mass-standardized peptides did not differ among predator treatments (LSD, all $p > 0.11$).

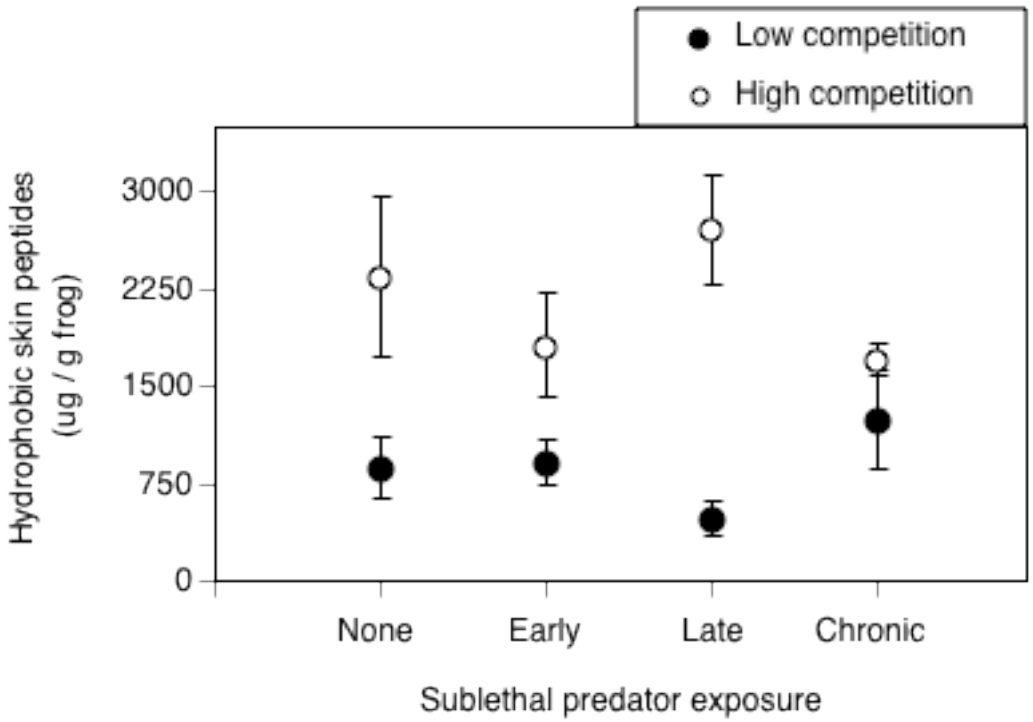


Figure 4.2. Effects of competition and predator cue treatments applied to leopard frog (*Rana pipiens*) tadpoles on their production of hydrophobic skin peptides nine days after metamorphosis. Analysis of variance showed that high competition significantly increased the production of peptides and that the timing and duration of predator exposure significantly altered the effect of competition.

Table 4.2. Results of an ANOVA examining the effects of larval competition and predator cue treatments on the amount of hydrophobic skin peptides produced by leopard frogs (*Rana pipiens*) 9 d after metamorphosis. For each response variable, *F*-values are listed first followed by *P*-values in parentheses.

Effect	df	Mass-standardized peptides
Model	(7, 24)	5.8 (< 0.001)
Competition	(1, 24)	32.3 (< 0.001)
Predator cues	(3, 24)	0.008 (0.943)
Competition* Predator cues	(3, 24)	3.7 (0.067)

4.4.3 Peptide characterization

Mass spectrometry showed at least six multiply charged peaks corresponding to the following monoisotopic masses 1426.2, 1875.2, 2568.6, 2592.9, 2622.0, 2877.0 (Figure B.1, B.2, Table B.1, B.2). Tandem mass spectrometry showed that all of these ions were consistent with being peptides, however, not all could be fully sequenced from the spectra that we obtained. In many cases we suspect that cysteine in the sequence yielded c-terminal cyclization via disulfide bonds (a common structure in AMPs) and prevented sequencing towards the carboxyl-terminal using our methodology. As would be predicted by such structures MS/MS spectra showed strong sequence signal, followed by an abrupt loss of signal (Kinter and Sherman 2000). Despite incomplete sequences the sequence obtained was sufficient to categorize these peptides into appropriate families.

Similarity with known brevinins for five of our peptide led us to conclude that our frogs were also producing brevinins (Table B.1). Currently, there are nine identified brevinin peptides for *Rana pipiens* (Table B.1), however our results suggest that at least four more exist, though this cannot be confirmed without full amino acid sequences. These four partial-sequences are nearly or completely homologous with known brevinin sequences; however, different molecular weights suggest that they may differ in sequence towards the carboxyl end, which we were unable to sequence (Figure B.2). Collection of more concentrated peptide samples from adult frogs in this area and sequencing following reduction to break disulfide bonds and alkylation with iodoacetamide to block disulfide reformation would be used to fully sequence these peptides. The low concentrations of peptides in our samples did not yield enough sample to modify our technique. The fifth sequence is likely brevinin-1Pd as both the sequenced amino acids and molecular weight are homologous with this interpretation.

We also sequenced a novel temporin, temporin-2P (Figure B.1). This is the second temporin that has been identified in leopard frogs. The spectra for this peptide was consistent with a c-terminally amidation, which is compatible with known temporins (Table B.2). Moreover, hydrophobicity and hydrophilicty were nearly homologous (84%) with the published temporin consensus sequence (Wade 2010).

4.5 DISCUSSION

This experiment tested the effects of competition and the timing and duration of exposure to predator-cues on life history traits and the production of skin peptides in amphibians. We found that overall, competition induced the release of more skin peptides; however this effect depended upon the nature of the exposure to predator cues. The positive effect of competition on skin peptides was masked or reduced when tadpoles were exposed to predator cues chronically or early in development. In contrast, exposure to no cues or late cues caused more than a 250% increase in skin peptide production in the high competition treatments. At low competition, the late-cue treatment actually caused a decrease in peptide production. This research, which is only the second study of the effects of both competition and predator-cues on immune functions in amphibians (Raffel et al. 2010), shows that predator stresses experienced during critical periods early in development or chronically throughout development can impair immune function during highly susceptible life history stages later in development, however others, such as competition, can enhance immune function.

Both competition and predator-cues altered life history traits of leopard frogs. Tadpoles

reared in high competition environments had decreased survival, growth and development. Survival in high competition treatments was reduced by 19% relative to low competition treatments. Much of this mortality occurred in the last 4 wks of the experiment (i.e. after experiment day 74, personal observation), so reduced survival did not have a large effect on the competitive environment for most of the experiment. Of the tadpoles surviving in the high competition treatments, only 80% developed enough to metamorphose before the experiment ended (Table 1). Many ponds dry in late summer, so retarded development can lead to death, if tadpoles cannot escape a drying pond by metamorphosing. These results are consistent with past studies that show negative effects of competition on the life history traits of frogs (e.g., Travis 1984, Werner 1986, Relyea and Hoverman 2003, Benard 2004, Relyea 2007).

Exposure to predator cues altered the sizes of metamorphosing frogs in both the early- and late-cue treatments, but not in the chronic-cue treatment. The lack of an effect of predator cues on survival and development time is consistent with past studies (reviewed in Benard 2004, Relyea 2007). While exposure to predators often alters developmental and growth trajectories, so that exposed tadpoles may be less developed and smaller than unexposed tadpoles mid-way through larval development, this is compensated for with faster growth and development closer to metamorphosis. The majority of studies of effects of sublethal exposure on tadpole growth have examined chronic exposure and found no effect (consistent with this study, Relyea 2007). To our knowledge, no other studies have examined the effects of short pulses of stress exposure on amphibian life history. It is interesting that both early-cue and late-cue treatments resulted in larger frogs at metamorphosis whereas the chronic-cue treatment did not. While it has been shown that the magnitude of plasticity in morphology and behavior is sensitive to the concentration of predator cues, a tadpole's ability to adjust plastic responses based on the timing

of exposure appears to be more limited (Schoeppner and Relyea 2008, 2009). We suspect that faster growth of tadpoles exposed to short pulses of predator-cues results from a plastic response that was adapted for a longer exposure to predator-cues than these tadpoles experienced.

Decreased growth and development rates in tadpoles are often considered indicators of lower fitness in amphibians because they have been correlated with decreases in adult survival, growth rates and size at reproduction (Howard and Kluge 1985, Smith 1987, Semlitsch et al. 1988, Altwegg and Reyer 2003, reviewed in Relyea 2007). However, the costs of altered life history may be quite different in the presence of virulent pathogens. Depending upon local host and pathogen phenologies, infection risk may decrease or increase as result of these life history changes (Biere and Honders 1996, Norris and Evans 2000, Zera and Harshman 2001, Hawley and Altizer 2010). For example, competition could increase exposure to water-borne pathogens as a result of slower development and increased densities. At the same time, the risk of infection by pathogens that infect metamorphs may decrease if tadpoles metamorphose later and delay their exposure to these pathogens (e.g., Raffel et al. 2010). The timing of host emergence relative to infection risk depends on both seasonally-driven factors such as temperature and pH which can affect growth rates of amphibians and their pathogens as well as biotic factors such as host community composition and life history which effect ratios of susceptible and infected hosts (e.g., Piotrowski et al. 2004, Rojas et al. 2005, Altizer et al. 2006, Hawley and Altizer 2010).

Competition increased the amount of hydrophobic peptides on amphibian skin. Despite having a smaller size, metamorphs raised in high competition produced, on average, 53% more skin peptides per unit mass than metamorphs raised in low competition. This result is contrary to our prediction that the stress of competition would negatively affect skin peptide production as a result of reduced allocation or acquisition of resources. Several mechanisms may explain these

patterns. Increased AMPs at high competition could result from increased synthesis of AMPs, a reduction in the frequency of AMP releases, so that granular glands had higher stores of AMPs during release or altered sensitivity to norepinephrine, which induces the release of peptides stored in granular glands. Perhaps the simplest explanation for increased peptides in animals raised at high competition is that they had more time to build peptide stores before they metamorphosed (~15 days later than tadpoles in low competition).

Theoretical predictions support patterns of both increased and decreased immune functions at high host densities. Lochmiller (1996) suggested that increased releases of stress hormones at high competition should cause immunosuppression; however transmission and the risk of disease increases with increased host densities (for pathogens with mass-action transmission), so increased allocation to costly immune defenses may provide the greatest fitness benefits at this time (Carey et al. 1999, Svensson et al. 2001). The few empirical tests of these hypotheses have mixed results. For example, density-dependent immunosuppression has been found in lizards (antibody-responsiveness, Svensson et al. 2001), while density-dependent immunoenhancement (in the form of increased melanism) has been found in moths (Hagen et al. 2006). No density-dependent effects on acquired immune functions (leukocyte count and type) were found in a recent study of in American toad (*Bufo americanus*; Raffel et al. 2010). Our results support the density-dependent immunoenhancement hypothesis, however we cannot tell if this pattern results from adaptive plasticity or is simply a consequence of altered life history patterns.

The effect of predator-cues on the mass-standardized production of skin peptides depended on the timing and extent of their exposure to predators as well as the competitive environment. We predicted that early acute stress would have more detrimental impacts on the

production of AMPs than late acute stress. Indeed, we found that exposure to early predator stress reduced immune responses in high competition, even though these immune measurements were taken ~7 weeks after exposure to this stressor ended. The early-cue treatment caused a marginally significant 2-fold increase in skin peptide concentrations in high competition, while the late-cue treatment caused more than a five-fold increase of skin peptides at high competition; this increase is due both to a (non-significant) increase in production of skin peptides at high competition and a decrease in production of skin peptides at low competition relative to controls. Observed interactions between predator cues and competition for other amphibian traits (behavior, morphology and physiology) find that predator cues have stronger effects on tadpoles in low competition environments (Relyea 2004a). It is not surprising to see the same pattern here (in the late-cue treatment); the risk of predation is relatively higher when population sizes are small and competition is less severe, thus we would expect to see a higher sensitivity to predator-cues in this environment.

We also predicted that chronic stress would deplete skin peptides more than acute stress. Our results supported this hypothesis. Animals exposed to predator cues throughout their larval period were not able to increase peptide releases under high competition, as they did in the late-cue treatment, and tended to do in the early-cue treatment (though this was marginally non-significant). At low competition, exposure of tadpoles to chronic-cues did not alter skin peptides relative to tadpoles exposed to early-cues, but it did cause tadpoles in exposed to chronic-cues to release more peptides than tadpoles exposed to late-cues.

While, interpreting the mechanism underlying these patterns is challenging with only one measurement of AMPs across time, variation in the timing of exposure to stressors relative to development may explain these results. Past studies show that exposure to predators induced

releases of AMPs (Bevins and Zasloff 1990, Clarke 1997, Ramsey et al. 2010, chapter 3), thus we might expect that animals frequently exposed to predators likely release peptides more frequently leading to depletion of peptides in the granular glands. The time required for full recovery of skin peptides after depletion has not been evaluated for metamorphs of any species or for leopard frogs; in *Xenopus laevis* adults, recovery takes 25-35 d after injection with 20 nmol norepinephrine per gram frog (Ramsey et al. 2010). In the high competition treatments, peptide measurements occurred on average 43 d after the last exposure to predator cues. Thus, there would likely be time for individuals to recover from these releases. In low competition, however, measurements of skin peptides occurred on average 28 d after the last exposure to predator cues (for the late- and chronic-cue treatments). Thus, in low competition, we might expect that tadpoles exposed to early-cues would recover peptide stores, while those exposed to chronic- and late-cues may not. This explanation fits the results for the early- and late-cue treatments. In chronic-cue treatments, it is possible that after prolonged exposure, tadpoles were able to adjust peptide production or sensitivity to this stressor so that they could compensate for more frequent peptide releases.

Chemical analysis of hydrophobic peptides produced by leopard frog metamorphs show that they contain several types of brevinins and one temporin, both of which have important antimicrobial properties against pathogens of amphibians (Tables B.1 and B.2). Some of these characterized peptides are previously undescribed. Recent analyses of the gene coding for brevinins in leopard frogs provides evidence for several gene duplication events resulting in at least five loci encoding at least nine different brevinins (reviewed in Tennessen and Blouin 2010). Moreover, high rates of synonymous substitutions, but low allelic diversity relative to interspecies allelic differences suggest that that these genes have undergone recent positive

selective sweeps (Tennessen and Blouin 2010). Brevinin peptides in leopard frogs vary regionally, suggesting that they may be locally adapted to deter microbial threats (Tennessen et al. 2009). Recent growth inhibition studies show that different brevinins vary in their ability to inhibit Bd and the gram-positive bacteria *Staphylcoccus epidermidis* (Tennessen et al. 2009). Collectively, these data suggest that these peptides may offer important fitness benefits, potentially due to their impacts on skin parasitic and mutualist skin microbes.

Temporins have also been shown to inhibit Bd growth *in vitro*. The level of inhibition is dependent upon the ability to attach to the fungal cell membrane, the formation of α-helices after attachment (thought to be necessary for membrane disruption) and resistance against fungal proteases (Rollins-Smith et al. 2003). Temporin-2P is predicted to form α-helices, which would yield six hydrophobic amino acids on the same surface, indicating that it has strong membrane binding potential (Wang and Wang 2004). This is only the second temporin identified for leopard frogs. Further analysis should test if this temporin is only synthesized at certain ontogenetic stages and if it is restricted to a single geographic area. Given the abundance of new suspected AMPs identified in this study, we suspect that more local variation in AMP diversity is awaiting discovery.

This study did not examine the effects of competition and predator stress on disease dynamics *per se* and caution should be used in extrapolating effects of altered immune responses to disease resistance (Hawley and Altizer 2010). For example, altered immune responses can be compensated for with altered behavior or other functionally similar immune responses. Moreover patterns of reduced or enhanced immune responses may not always cross through thresholds that determine if an immune response is successful (e.g. chapter 3). Costly immune responses may ultimately cause more damage as a result of auto-immunity and build-up of allostatic load

(Lochmiller and Deerenberg 2000, McEwen and Seeman 2006). Finally, other effects of these stressors on life history traits, acclimation to future stressors and susceptibility to other threats may have greater influence on fitness than host immunity For amphibian metamorphs, many components of the acquired immune system are temporarily unavailable, so compensation for altered skin peptide production with functionally redundant immune responses may not be possible. Moreover, concentrations of peptides on amphibians are generally within the range that could inhibit skin infection with Bd (Ramsey et al. 2010). Thus, we cautiously suggest that the alterations of skin peptides found in this study as a result of competition and predator stress may have important consequences for the transmission and virulence of amphibian pathogens and their effects on host populations. In particular, we expect that animals reared at high densities and exposed to predators early in development or throughout development may have increased susceptibility to infection and disease.

4.6 CONCLUSIONS

Recent hypotheses have suggested that immunosuppression, resulting from altered environmental conditions may contribute to increased incidence of amphibian disease around the world. Our study shows that common environmental stresses can alter important immune defenses against pathogens that cause emerging infectious diseases in amphibians. In particular, we found that stresses experienced early in development or chronically can lead to decreases in the amount of antimicrobial peptides released in response to NE injection. These decreases were most apparent when coupled with another stressor, competition; however, contrary to our

predictions, competition actually increased overall production of skin peptides. We suspect that many of these effects can be explained by the timing of exposure to stress relative to metamorphosis. In general, animals that had more time to recover from exposure to a stressor, or had a longer development had greater stores of peptides. Further studies are needed to assess the costs of increased antimicrobial peptide production and whether these changes are adaptive.

5.0 HEALTHY HERDS AND TRAIT-MEDIATED EFFECTS: PREDATORS REDUCE INFECTION PREVALENCE AND INTENSITY OF BATRACHOCHYTRIUM DENDROBATIDIS IN A SUSPECTED RESERVOIR HOST

5.1 ABSTRACT

Disease ecologists and wildlife managers have been increasingly interested in understanding how predators regulate infection in prey populations. The healthy herds hypothesis suggests that predators may decrease infection prevalence by culling infected individuals from a population and also by decreasing overall population size, thereby reducing density-dependent transmission. Models such as these are based on density-mediated indirect interactions (DMIIs) and do not typically incorporate trait-mediated indirect interactions (TMIIs) of predators and pathogens into their predictions. However, it is recognized in many systems that TMIIs can be equally or more influential on community interactions than DMIIs.

In this experiment we examined TMIIs resulting from exposure to the fungal pathogen, *Batrachochytrium dendrobatidis*, and predator cues from larval Dytiscid predators. We measured effects of these stressors on infection prevalence and intensity and inducible anti-predator defenses in wood frog (*Rana sylvatica*) tadpoles. We found evidence that predators can alter host-pathogen interactions, and pathogens can alter predator-prey interactions. In both cases, these alterations would be predicted to reduce overall infection intensity and prevalence in

a population. Exposure to predators reduced the risk of Bd; tadpoles exposed to both stressors had reduced infection intensity, potentially as a result of stress-induced immunoenhancement. Infection with Bd increased the risk of predation while resistance to Bd decreased the risk of predation. This is because infection caused tadpoles to increase activity, which is associated with risk of predation, while resistance led to a decrease activity. These dual effects would be expected to reduce the ratio of infected to susceptible and resistant tadpoles in the presence of predators. Tadpoles exposed to Bd had faster development than tadpoles not exposed to Bd, suggesting that there are few costs of infection at this life stage; this supports the idea that tadpoles may be a reservoir host for this pathogen. Therefore regulation of infection prevalence in tadpoles as a result of TMIIs and density-mediated indirect interactions may be important for determining epidemiological outcomes of Bd in vulnerable populations.

5.2 INTRODUCTION

Recent efforts in conservation and wildlife epidemiology focus on the role of predators in controlling infections in host populations (Hudson et al. 1992, Packer et al. 2003, Duffy et al. 2005, 2011). The healthy herd hypothesis (Packer et al. 2003) suggests that for density-dependent pathogens, predators should decrease absolute and relative numbers of infected prey. This is predicted to occur both because predators can reduce host densities, which reduces pathogen transmission, and because predators are often more likely to cull infected individuals (Packer et al. 2003). Empirical evidence shows mixed support for the healthy herds hypothesis. In some cases predators reduce infection prevalence (Hudson et al. 1992, Lafferty 2004, Duffy et

al. 2005, Johnson et al. 2006). However, other studies find positive correlations between predator densities and infection prevalence (Cáceres et al. 2009, Hawlena et al. 2010, Duffy et al. 2011). This diversity of outcomes suggests that additional mechanisms may influence the strength and direction of these multi-enemy interactions.

While the healthy herd hypothesis is focused on the role of predators in controlling pathogen prevalence in their prey, an equally important question is how infection alters the role of predators in maintaining or reducing prey populations. For example, if infection or resistance to infection increases susceptibility to predation, this could cause predators to have greater top-down control on prey populations, thereby reducing or eliminating prey populations and possibly causing concomitant reductions in their parasites (e. g. Duffy et al. 2005, Johnson et al. 2006, reviewed in Johnson et al. 2010). Infection is often accompanied by changes in traits (e.g. behavior, appearance and growth) that would be expected to change predation risk. Increasing our understanding of how these trait changes influence predator-prey-parasite interactions should improve our understanding of the diversity of outcomes resulting from these interactions in natural systems.

An additional factor that could improve our understanding of these interactions is a greater focus on trait-mediated indirect interactions (TMIIs). Traditionally, predator-prey-pathogen models focus exclusively on density-mediated indirect interactions (DMIIs) of predators on disease dynamics (e.g., Packer et al. 2003, Ostfeld and Holt 2004, Holt and Roy 2007, Roy and Holt 2008). These effects can change patterns of between-host infection dynamics by altering population densities and ratios of infected to susceptible individuals. However TMIIs of predators and pathogens may also play a crucial role in determining pathogen-host and predator-prey interactions (Miner et al. 2002, Keesing et al. 2006). In the case of predators,

TMIIs occur when an organism can detect the predator and respond by inducing plastic defenses that decrease the risk of predation (reviewed in Miner et al. 2002, Relyea 2003b, Auld et al. 2010). These defenses can be behavioral, morphological, physiological and life historical (e. g. Benard 2004). Similarly pathogen hosts can induce behavioral, morphological and immunological defenses against infection (Martin 2009). In predator-prey-pathogen systems, if these phenotypic changes fundamentally alter host-pathogen or predator-prey interactions then these TMIIs may be an important mechanism that should be added to the healthy herd hypothesis. While they are relatively unexplored in predator-prey-pathogen systems, TMIIs in other systems can have stronger effects on communities than DMIIs (Werner and Peacor 2003).

One might expect TMIIs among predators, prey and their pathogens for several reasons. First, inducible defenses alter victim-enemy interaction by reducing the impact of the enemy. In addition, inducible defenses are often costly to produce (reviewed in Dewitt et al. 1998, Auld et al. 2010). Therefore, induction of plastic defenses may have associated trade-offs that increase vulnerability to other threats (i.e. risk enhancement). For example, tadpoles often have reduced anti-predator defenses at high competition and reduced competitor-induced defenses (i.e. morphology) under high predation risk (e.g., Relyea 2004a, Relyea and Auld 2005). Similar trade-offs have been seen in inducible defenses against multiple predators (e.g., Poitrineau et al. 2003, Hoverman and Relyea 2009, reviewed in Relyea 2003a, Sih et al. 1998). In the case of predator-prey-parasite systems, risk enhancement could lead to increased predation risk in populations that exposed to pathogens or increased infection risk in populations that are exposed to predators. Alternatively, inducible defenses may cause risk reduction in the face of another threat if inducible defenses reduce the risk of both threats (Sih et al. 1998). For the predator-prey-parasite systems, this implies that 1) populations exposed to predators experience reduced

costs due to pathogens than populations not exposed to predators and 2) populations exposed to pathogens experience reduced costs of predators than populations not exposed to pathogens. Both risk reduction and risk enhancement could alter susceptibility to infection and predation. Despite the potential for TMIIs to change how and when predator and pathogen populations regulate each other, few studies evaluate how prey phenotypes change in these multi-threat environments and if those changes influence these multi-trophic interactions (Lefcort and Blaustein 1995, Navarro et al. 2004, Thiemann and Wassersug 2000, Keesing et al. 2006, Parris et al. 2006, Martin 2009, Han et al. 2011).

One taxonomic group that could benefit from these studies is amphibians. Emerging infectious diseases (EIDs) are having a devastating impact on many amphibian species around the globe (reviewed in Collins and Storfer 2003, Daszak et al. 2003, Stuart et al. 2004, Skerratt et al. 2007, Fisher et al. 2009, Kilpatrick et al. 2010). Along with a number of other biotic and abiotic factors, EIDs are contributing to the decline of over 40% of amphibian species around the globe (reviewed in Daszak 2003, Collins and Storfer 2003, Beebee and Griffiths 2005, Blaustein et al. 2011). For example, the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) is linked with amphibian population declines or extinction at locations across five continents, while having no or marginal effects in other locations, sometimes for the same species (Lips et al. 2006, Kriger et al. 2007, Tobler and Schmidt 2010, Vredenberg et al. 2010, reviewed in Fisher et al. 2009). It has been hypothesized that exposure to environmental stressors may be facilitating Bd infection (e.g. Carey et al. 1999, Fisher et al. 2009, Blaustein et al. 2011, Rollins-Smith et al. 2011). We understand how a number of abiotic factors such as temperature, altitude and moisture correlate with Bd prevalence (Retallick et al. 2004, Ron 2005, Pounds et al. 2006, Bielby et al.

2008), but we know very little about how community composition and multi-trophic interactions affect Bd infection and persistence (Parris et al. 2004, 2006, Han et al. 2011, Buck et al. 2011).

Limited evidence suggests that predator-cues can cause both risk enhancement and risk reduction in the presence of Bd (chapter 3 and 4). Many larval amphibians are exposed to a wide range of invertebrate predators (e.g. Wellborn et al. 1997) and they possess a remarkable variety of well-described behavioral, physiological, morphological and developmental inducible responses to predators (reviewed in Relyea 2003a, Benard 2004). Exposure to predators can reduce an innate immune response (release of antimicrobial peptides onto the skin) in recently metamorphosed Ranids (chapter 3 and 4) that is associated with resisting Bd infection (reviewed in Rollins-Smith and Conlon 2005, Ramsey et al. 2010, Rollins-Smith et al. 2011). Exposure to predators has also demonstrated risk reduction in metamorphosed frogs, because wood frogs metamorphs exposed to Bd were able to live longer with an infection, despite this reduction in immune function (chapter 3). As far as we known, there are few studies exploring how predators alter host-pathogen interactions in tadpoles exposed to Bd (Parris et al. 2004).

Exposure to Bd may also change susceptibility of amphibians to predators as a result of risk enhancement or risk reduction. For example, tadpoles exhibit a range of responses to infection; some tadpoles show no costs of infection and others exhibit altered behavior and morbidity (Parris et al. 2004, 2006, Blaustein et al. 2005, Venesky et al. 2009, Han et al. 2011). These behavioral changes have been correlated with both decreased and increased predation rates (Parris et al. 2006, Han et al. 2011). It is unclear, however, and if these changes are due to infection itself, or immune responses against infection. Moreover it is unknown if Bd alters other inducible defenses against predators.

For many amphibians, the larval stage may be especially important for Bd dynamics. Tadpoles often serve as a reservoir host for Bd (Daszak et al. 1999, 2003), experiencing few costs of infection (Haydon et al. 2002, Rachowicz and Vredenburg 2004, Blaustein et al. 2005). Reservoir tadpoles may maintain Bd presence in a population, and transmit Bd to other amphibians that are in more vulnerable life stages (Blaustein et al. 2005). Therefore reductions in Bd prevalence among tadpoles, as a result of environmental conditions including predation risk, should reduce infection prevalence throughout an amphibian community. While not well explored (Parris et al. 2006, Han et al. 2011), evidence suggests even mild infection with Bd can change tadpole behavior. This has the relatively-unexplored potential to alter how tadpoles interact with their predators, which could influence how predators regulate both amphibians and their pathogens.

In this experiment, we tested if exposure to predators altered susceptibility to infection and if exposure to pathogens altered inducible defenses against predators. We exposed wood frog tadpoles (*Rana sylvatica*) to caged predator and no-predator environments crossed with the presence or absence of Bd, and tested how these treatments affected inducible defenses against predators and infection rates. We predicted that 1) sublethal exposure to predators would alter the incidence of Bd infection and the intensity of the infection, 2) exposure to Bd would alter induction of morphological and behavioral defenses against predators, 3) induction of defenses against predators would be costly, leading to reduced growth and development of tadpoles and 4) infection and resistance to infection would be costly, leading to reduced growth and development of tadpoles.

5.3 METHODS

5.3.1 Experimental Design

The experiment was conducted between 12 May (experiment day 1) and 2 June 2010 at the Pymatuning Lab of Ecology in northwest Pennsylvania, USA. We used a completely randomized full-factorial design crossing two predator treatments with two infection treatments. Each of the four treatments was replicated 69 times for a total of 276 experimental units. Experimental units were 1-L containers filled with 600 mL of charcoal filtered, UV-radiated well water (pH = 8). Each container held one wood frog tadpole (Gosner stage 26, Gosner 1960, mass: 44.8 ± 2.7 mg). The tadpoles came from a mixture of ten egg masses that were collected from a single pond on March 31 and reared in outdoor mesocosms until the start of the experiment. Outdoor mesocosms were covered to prevent colonization by flying predators. Experimental units were kept indoors at 21 °C on a 14:10 light:dark schedule. Replicates were arranged into two spatial blocks (i.e. two shelf heights). Water was changed every 4 d and tadpoles were fed 4% of their body mass every 2 d in ground Tetramin® fish food.

Predator cue treatments consisted of daily exposure to predator cues or sham cues. Predator cues came from locally collected predacious diving beetle larvae (*Dytiscus spp.*). Cues from dytiscid beetle larvae in particular induce lower activity (Van Buskirk 2000, Relyea 2001, 2002, Schoeppner and Relyea 2008) and shorter and deeper tails and bodies in wood frog tadpoles (Relyea 2001, 2002, Schoeppner and Relyea 2008). While these defenses reduce predation risk, they have a cost of reduced foraging, and in some cases, reduced growth and development (Skelly 1992, 1994, reviewed in Relyea 2007).

Predator cues consisted of water taken from a tub containing Dytiscids that were fed wood frog tadpoles. Digested and excreted conspecifics are known to induce a fear response in tadpoles, thus allowing us to study sublethal effects of predators animals without losing focal individuals. Predators were housed separately in 200-mL cups with mesh lids. These cups were floated in a single 14-L container holding 10-L of well-water. Each predator was fed 2 g of wood frog tadpoles every 2 d. Water from the predator container (15 mL) was added to the appropriate replicates daily, producing a concentration of 5 mg of digested tadpole/ L of water in the predator-cue treatments. Tadpoles in the no-cue treatment received 15 mL of well-water daily. In mesocosm experiments that contain caged predators, 2.3 mg digested tadpole/ L delivered 2 d is a high enough concentration to elicit maximum plastic responses (Schoeppner and Relyea 2008). Even though our cues had a concentration more than 2 times as great, we suspect that some of the predator cue degraded before we dosed the containers, because we did not observe tadpoles responding behaviorally to these concentrations. Therefore, on day 19, we tripled the concentration of the cue (by tripling the mass of tadpoles fed to the Dytiscid predators). Survival of tadpoles was assessed daily during administration of predator cues.

Bd-exposure treatments consisted of exposure to Bd zoospores or exposure to a Bd-free control. To create these treatments we cultured Bd isolate JEL258 (from a wood frog collected in Orono, ME) on 1% tryptone agar plates. This isolate was cryopreserved between the initial isolation and use in our experiment to minimize attenuation of virulence across generations. After 6 to 8 d of culturing, plates were flooded with 3 mL of well-water and left for 15 to 30 min to allow sporangia to infective zoospores. We then gently scraped the plates to detach cells from the agar. We pooled the suspension from all plates and quantified zoospore abundance with a hemocytometer. The liquid was then diluted to a concentration of ~10^6

zoospores/ mL. Tadpoles in the Bd treatment received 1 mL of this mixture every 4 d (after water changes). We chose to use this concentration of zoospores so that we could be sure that we reached a threshold concentration necessary to achieve infection (Searle et al. 2011). Control animals received an equal amount of water that was collected from sterile 1% tryptone agar plates. To minimize cross-contaminating animals, all equipment used during the experiment was sterilized with a >10% bleach solution immediately after use in each replicate (Johnson et al. 2003). The experiment was terminated after 23 d.

5.3.2 Response variables

To understand how predator cues and Bd exposure alter inducible behavioral responses, we measured the activity of tadpoles on days 8, 14, 19 and 22. Activity measurements were made by observing each container for ~2 sec and recording if the tadpole was moving or not. This was repeated 6 to 8 times on each day that activity measurements were made. Activity measurements were taken prior to daily administration of predator cues (i.e. 23 hrs after adding the previous cue). However, after finding little evidence of predator effects on activity on days 8, 14 and 19, we suspected that the cues were breaking down before we measured activity. We switched our measurement for day 22 to 1 hr after the administration of predator cues.

Infection prevalence (the proportion of individuals infected) and infection load (the amount of zoospores on an individual) was determined using quantitative PCR. At the end of the experiment, (i.e. day 23), each animal was swabbed 30 times along keratinizing tissue using sterile swabs (Medical Wire & Equipment Co., MW 100-100). Bd only infects keratinizing

tissue, which for tadpoles consists of their mouthparts. After swabbing, we euthanized tadpoles in MS-222 and preserved them in 10% buffered formalin.

Swabs from all tadpoles in the Bd-exposure treatments and 45 randomly selected tadpoles in the Bd-free treatments were analyzed for infection load. We used a slightly modified version of the quantitative PCR protocol outlined in Boyle et al. (2004); we used 50 uL of Prepman Ultra (Applied Biosystems) for the DNA extraction (as oppose to 40 uL) and we did not bead-beat the swabs during DNA extraction. Quantitative PCR samples for each swab were run in triplicate and were re-run in cases where the assay yielded only one or two positive results. A sample was considered positive if at least three of six wells tested were positive. Such variability between wells is common in samples with low infection levels. Quantitative estimates of infection loads were made by averaging the positive results for replicate wells. Of the 45 samples from the Bd-free treatment, only one sample tested positive for Bd, suggesting that cross-infection was rare.

To determine whether Bd could affect tadpole morphology or interact with the predator induction of tadpole morphology, we measured the final morphology of all the tadpoles in the experiment. Morphological measurements were taken from on lateral images of preserved animals. We placed tadpoles on their sides and propped their tails on top of slides so that they were in line with the tadpole body. We then took lateral photos of these tadpoles using the software DP2-BSW (Olympus 2008). This software has a digital scale bar that we used calibrate our images. Seventeen landmarks were marked on imaged tadpoles that represented biologically important features or mid-points between biologically important features (ImageJ 1.43u, Figure 5.1). Measurements of landmarks had high repeatability. The average product moment correlation for repeated measurements of each landmark (n=10) was 0.991.

To assess the whether exposure to predator cues or Bd posed any costs, we quantified the mass, developmental stage (Gosner 1960) and survival of tadpoles. We assessed survival each day of the experiment.

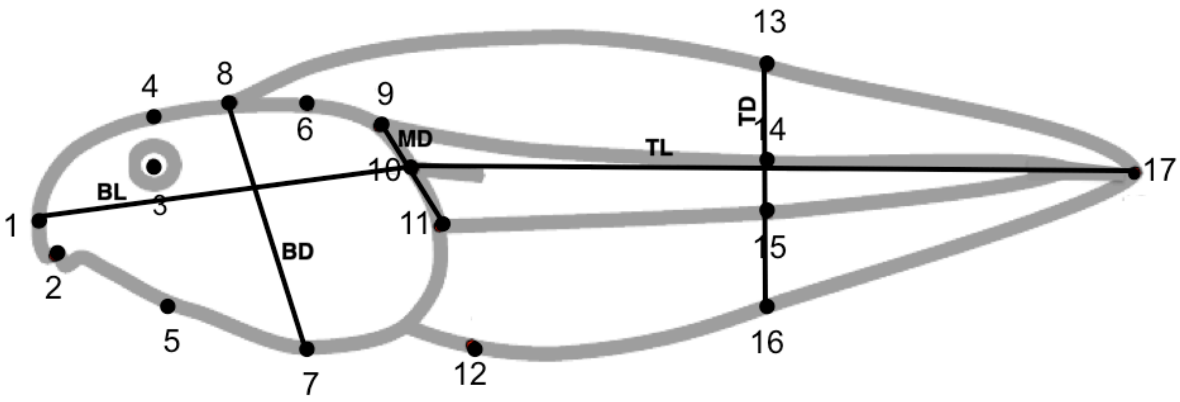


Figure 5.1. Morphological landmarks on lateral images of tadpoles. Most landmarks represent distinct morphological features. To increase resolution of the head and tail shape, points 4, 5, 6,7 and 13, 14, 15 and 16 were included. Points 4 and 5 create a line perpendicular to the line made by 1 and 11 and bisect the eye. Points 6 and 7 also form a line perpendicular to line 1-11, bisecting it at two thirds of its length. The line created by 13, 14, 15 and 16 is perpendicular to line 10-17 and bisects it halfway. Linear dimensions for tadpoles are also shown. BL = body length, BD = body depth, MD = muscle depth, TD = tail depth, TL = tail length.

5.3.3 Statistical Analyses

5.3.3.1 Infection status and infection load

We used a zero-inflated negative binomial regression with a log-link to test the effects of both block and predator cues on the infection status and the zoospore load of an individual. This method is useful in analyzing count data that have a high frequency of zeros. Moreover it is biologically interpretable because it simultaneously evaluates the probability of no event (e.g., resistance to an exposure) and the intensity of the events that occur (e.g., infection load). Since only about 30% of tadpoles exposed to Bd tested positive for infection, we used this model to test how treatments affect both whether an individual was infected and, if so, how many zoospores it had. This analysis was done in R (v. 2.13.0) using the zeroinfl command in the package pscl.

5.3.3.2 Tadpole activity

We used a generalized linear mixed model with a binomial distribution and a logit-link to test the effects of time, block, predator cues and exposure to Bd on tadpole activity for the first three activity measurements. In many of the activity trials tadpoles never moved, leading to an excess of zeroes in our dataest. Therefore we included a random intercept with a normal distribution in the model because it can account for excess zeros in a dataset. This provides a more direct interpretation than other methods used to account for zero-inflated distributions (Min and Agresti 2005). Because of the large number of terms and interactions in this model, we used a model selection approach to pick the best model. First we used forward selection to add terms that improved the model fit. We then used backward selection to see if terms could then be dropped

out of the model without losing explanatory value. For both procedures, we picked terms based on Akaike information criteria values, -2 log-likelihood scores and p-values. Our final model had a Pearson χ^2 /degrees of freedom of 1.07, signifying that little variation in the activity data was not explained by our model. These analyses were done using SAS v. 9.3.

We analyzed the last activity measurement separately because we had changed the predator cue concentration and the timing of activity measurements. To do this, we also used a generalized linear model with a binomial distribution and a logit-link. We did not perform model selection on this final model, however, since we had fewer terms in the model (i.e. time was not a factor in the model). These analyses were done using SPSS v. 18.0).

Since not all of the tadpoles that were exposed to Bd were infected at the end of the experiment, we wanted to test if this was due to unsuccessful application of the experimental treatment or resistance to infection. To do this, we compared the activity of uninfected, Bd-exposed tadpoles with tadpoles that were not exposed to Bd (including predator and block in the model). Because we saw differences between these two treatments, we concluded that the treatments were applied correctly. We used this analysis to evaluate the costs of resisting an infection relative to not having an infection. To understand the costs of carrying an infection relative to resisting an infection (the infection model) we repeated the analysis, but excluded the no Bd control from the experiment and compared Bd-positive to Bd-negative tadpoles. For simplicity, the original model design will be referred to as the exposure model and the additional tests will be referred to as the resistance model and the infection model.

5.3.3.3 Tadpole morphology

We used both geometric morphometric analysis and analysis of linear tadpole dimensions to test for treatment effects on tadpole morphology. For the geometric morphometric analysis, we first had to remove size differences from landmark measurements. Landmarked images of lateral views of tadpoles were rescaled to equalize centroid size. Rescaling was done using only landmarks on the head because tail landmarks, due to their increased distance from other landmarks would have too much leverage on these adjustments. After removing the effect of shape, we aligned the morphometric data using a least-squared generalized Procrustes imposition to minimize distances between corresponding landmarks (reviewed in Zelditch et al. 2004).

We then analyzed the effects of predator cues, Bd-exposure, block and their interactions on the adjusted morphometric data using permutation MANOVA. Since developmental stage also affects tadpole morphology and might influence responses to infections (Walker et al. 2010), we also included Gosner stage and its interactions with other model terms in the model. All terms were considered fixed effects. We excluded seven individuals from the analysis that had extremely retarded or accelerated development (e.g., Gosner stage 33 or 39) as well as several outliers (determined using MorphoJ, 'find outliers' function, Klinenberg 2011). The MANOVA was performed in R (v. 2.13.0) using the adonis command in the package vegan. For each analysis, the raw data was permuted 10,000 times. As in the activity analyses, geometric morphometric analyses were repeated two additional times in order to evaluate the effects of Bd resistance and infection on tadpole morphometrics.

We tested for pairwise differences where model terms were significant using permutation MANOVA (vegan package R, v. 2.13.0). Since this package will not do pairwise comparisons

itself, we first adjusted the data to control for the significant main effects before running pairwise comparisons. Multiple comparisons were corrected with a sequential Bonferroni adjustment (Holm 1979). We then used the software program MorphoJ (Klinenberg 2011) to visualize pairwise differences between treatments (using the graphical component of the discriminant function analysis).

Most morphometric analyses of tadpoles have not used geometric morphometric analyses, but instead focus on specific linear dimensions (reviewed in Relyea 2003a). We also analyzed our data with this method so we could compare our results with other papers and to see how geometric morphometric analyses compare with this alternative method. To do this, we measured tail length and depth, muscle depth, and body length and depth (see figure 1). We analyzed this data with a multivariate analysis of covariance (MANCOVA). To control for the effect of size on morphometric measurements, we used $\log_{10}$ mass as a covariate, while analyzing the effects of block, Bd-exposure, predator-cues and their interactions on these traits. To meet the assumption of homogeneous variances and normality of residuals, we log-transformed all linear dimensions. MANCOVAs also assume that the covariate does not interact with treatment effects (White 2003). Our data violated this assumption. Therefore, we used the Johnson-Neyman method to identify the range of the covariate treatment that treatment effects or interactions occurred (Johnson and Neyman 1936). We used a sequential Bonferroni to adjust for multiple comparisons for this analysis (Holm 1979).

5.3.3.4 Tadpole growth, development and survival

We used ANOVA to test the effects of predator cues, Bd-exposure, block and their interactions on tadpole mass. All factors were treated as fixed effects. Mass data met the assumptions of the

model without transformation. When interactions were significant, we made pairwise comparisons to understand these effects. We used a sequential Bonferroni to correct for multiple comparisons.

Since Gosner developmental stages are ordinal but not scalar, we used an ordinal logistic regression with a probit-link function to test the effects of predator cues, Bd-exposure and block on development. Since we were also interested in the effects of resistance to Bd and Bd infection on mass and development, we repeated these analyses two additional times using the resistance and infection models (described above).

We analyzed survival with a Cox's proportional hazards model (Cox 1972) with Bd, predator cues, block treatments and interactions as fixed factors. Data met the assumption of proportionality. This analysis was run in SPSS (v. 18.0).

5.4 RESULTS

5.4.1 Infection status and infection load

Exposure to predators did not alter infection prevalence, but it did cause infected tadpoles to have lower infection loads. The zero-inflated portion the model showed no effect of predator or block on whether a tadpole was infected (both $p > 0.31$). However, the log-link negative binomial portion of the model showed that exposure to predators decreased the pathogen load in tadpoles that were infected by nearly an order of magnitude ($p = 0.022$). Block was not significant in this model ($p = 0.74$). For this model, the dispersion parameter log (theta) was highly significant ($p = 0.011$), signifying that the negative binomial distribution was a good

approximation for these data. Of those animals exposed to Bd, 26% tested positive for infection in the no-predator treatment and 34% tested positive in the predator treatment. Among animals that tested positive for infection, tadpoles in the no-predator treatment had mean infection loads of 128 zoospore equivalents whereas tadpoles in the predator treatment had mean infection loads of 47 zoospore equivalents.

5.4.2 Tadpole activity

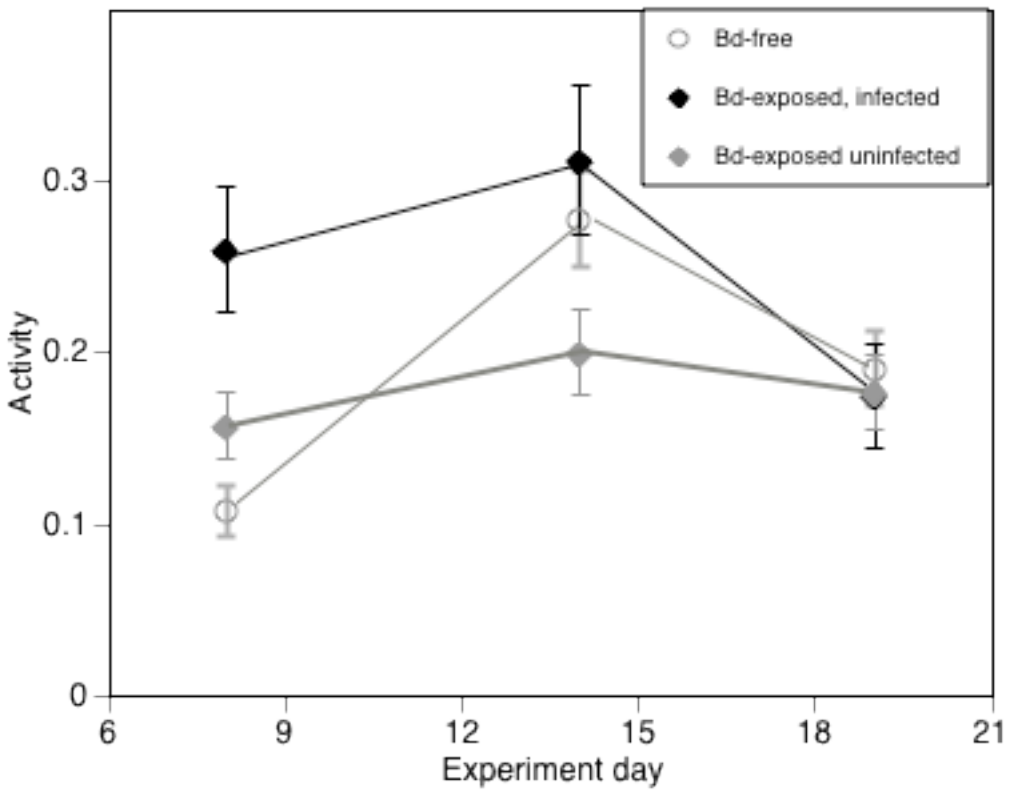


Figure 5.2. Effects of exposure to Bd and infection with Bd on the activity of wood frog tadpoles over 3 observation periods. Graphs depict probabilities of activity ± standard errors based on statistical modeling (described in text).

For the first three activity measurements, which were analyzed together, the full model showed significant effects of time, time-by-Bd and time-by-Bd-by-block on tadpole activity (Figure 5.2, Table 5.1). On day 8, tadpoles exposed to Bd had a 156% greater activity than unexposed tadpoles, while on day 14 they were 25% less active than unexposed tadpoles. On day 19 there was no difference between these groups. The interaction with block occurred because the affect of Bd on activity was stronger on the upper shelf of the experiment, especially on day 8 and 14.

The resistance model showed significant effects of time and the block-by-predator-by-Bd, time-by-block-by-predator, and time-by-block-by-Bd interactions (Figure 5.3, Table 5.1). The interactions with predator cues occurred because, in the presence of predator cues, unexposed tadpoles were 114% more active than predator-naïve tadpoles. In the presence of predator cues, uninfected, Bd-exposed tadpoles were 23% less active than predator-naïve tadpoles. This latter trend increased over time, while the former did not. The interaction with time, block and Bd occurred, because as in the previous model, Bd-exposure caused uninfected tadpoles to have higher activity than unexposed tadpoles on day 8, but much lower activity on day 14 and a slightly lower activity on day 19. The block term was part of all the significant interactions because activity was greater on the upper shelf of the experiment on day 14, but not on day 8 or day 19.

The tolerance model showed significant effects of infection, time, time-by-infection and time-by-block (Table 5.1). This occurred because infected tadpoles had a much higher probability of activity than uninfected, Bd-exposed tadpoles on days 8 and 14 and because tadpoles were more active on the upper shelf of the experiment on these days.

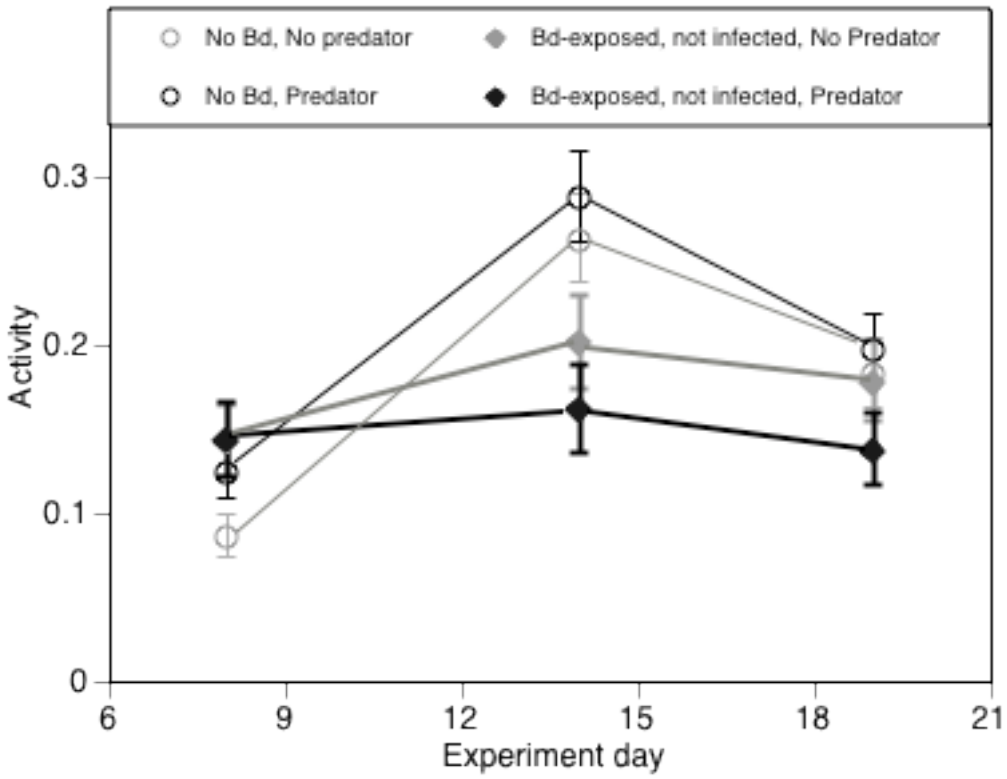


Figure 5.3. Effects of resistance to Bd and predator cues from larval Dytiscid beetles on the probability of activity in wood frog tadpoles on experiment days 8, 14 and 19. Exposed animals were considered 'resistant' if they were exposed to fungal spores yet tested negative for infection. About ~70% of tadpoles exposed to Bd were resistant to infection. Graphs depict probabilities of activity ± standard errors based on statistical modeling (described in text).

Table 5.1. Effects of predator cues and Bd on the activity of wood frog tadpoles . A) comparing effects of fungal exposure (e.g., the original treatments) on these traits. Because ~70% of animals exposed to Bd tested negative for infection at the end of the experiment, we tested if uninfected, Bd-exposed tadpoles (e.g., resistant tadpoles) were different from unexposed tadpoles activity (table 1b). Finally, because we saw differences between these two groups, we also tested if resistant and infected Bd-exposed tadpoles differed in activity (table 1c). Activity measurements were analyzed with two tests. Measurements taken on the first three trials (days 8, 14 and 19) were analyzed with a generalized linear mixed model with a binomial distribution, logit link and random intercept. Because of the number of terms in the fully-saturated model, step-wise model selection was used to pick the best model (presented here). F-statistics (for ANOVA) are shown with p – values in parenthesis. Values significant at $p < 0.05$ are shown in bold.

A.

Predator cues (P)	Block (B)	Bd exposure (E)	Time (T)	T*E	T*B	T*B*E
	3.19	0.01	**32.6**	**15.12**	1.66	**3.91**
	(0.0746)	(0.9128)	**(< 0.0001)**	**(< 0.0001)**	(0.1906)	**(0.0207)**

B.

Predator cues (P)	Block (B)	Bd Resistance (R)	P*B	P*R	B*R	Time (T)	T*R	T*B	T*P	T*B*P	T*B*R
0.01	1.04	1.28	0.19	2.63	0.00	**24.88**	**9.61**	1.43	1.44	**3.38**	**3.72**
(0.9399)	(0.3082)	(0.2583)	(0.6643)	(0.1059)	(0.9568)	**(<.0001)**	**(<.0001)**	(0.2405)	(0.2379)	**(0.0351)**	**(0.0252)**

C.

Predator cues (P)	Block (B)	Bd Infection (I)	Time (T)	T*I	T*B
2.09	0.70	**5.96**	**5.59**	**3.60**	**4.83**
(0.1493)	(0.4022)	**(0.0154)**	**(0.0043)**	**(0.0289)**	**(0.0088)**

We analyzed the final activity measurements separately because we increased the concentration of the predator cues and made our behavioral observations much sooner after the predator cues were added. In the exposure, resistance and infection analyses, predator cues caused 25-30% decreases in activity (Wald $\chi^2_{df=1} = 9.129$, $p = 0.003$, Wald $\chi^2_{df=1} = 6.348$, $p = 0.012$, Wald $\chi^2_{df=1} = 3.281$ $p = 0.070$, respectively, Figure 5.4). For the exposure model there was also a significant effect of block (Wald $\chi^2_{df=1} = 6.489$ $p = 0.011$) and a marginal effect of Bd-by-block on activity (Wald $\chi^2_{df=1} = 3.655$, $p = 0.056$). This interaction occurred because on the upper shelf, Bd-exposed animals were slightly more active than unexposed animals and on the lower shelf, Bd-exposed animals were slightly less active than unexposed tadpoles. In other words, exposure to Bd reduced the effect of block on activity. The block effects occurred because tadpoles on the lower shelf had about a 40% greater activity than tadpoles on the upper shelf. The resistance model also had a significant effect of block due to this pattern (Wald $\chi^2_{df=1} = 6.151$, $p = 0.013$). There were no other significant terms in the tolerance model.

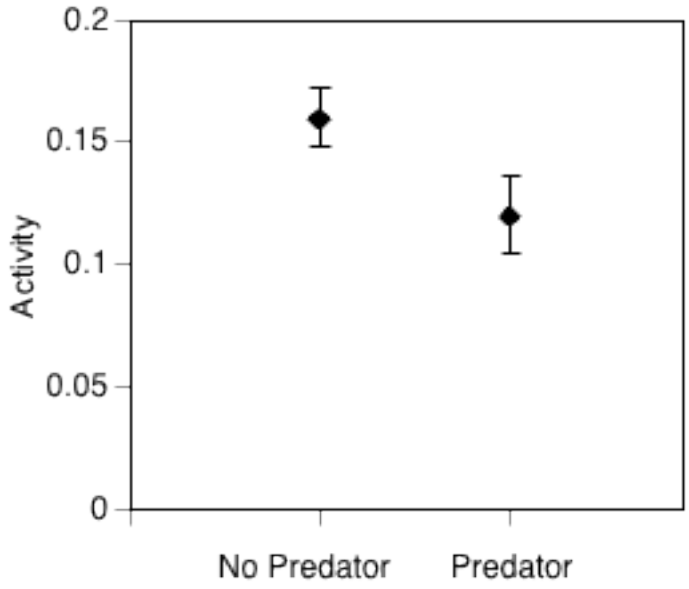


Figure 5.4. Effects of exposure to caged predators (larval Dytiscid beetles) on the probability of activity in wood frog tadpoles on experiment day 22. Graphs depict probabilities of activity ± standard errors based on statistical modeling (described in text).

5.4.3 Tadpole morphology

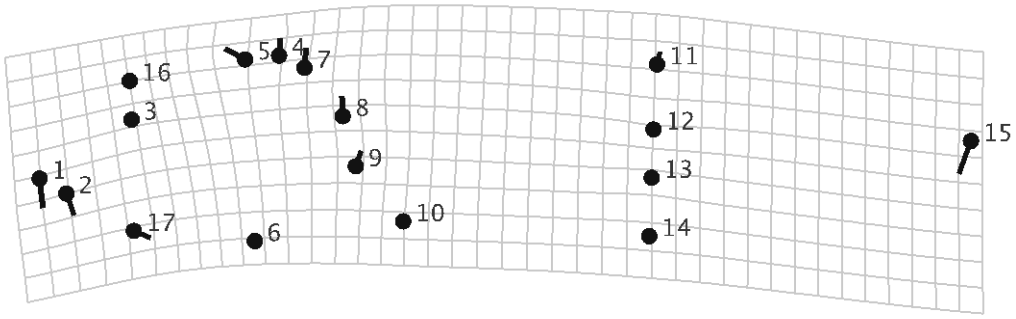


Figure 5.5. Effect of predator cues on wood frog morphology. Circles represent the mean landmark locations for unexposed tadpoles, while lines point towards shape change caused by exposure to predator-cues. Images were created in MorphoJ (v. 1.03a, Klinenberg 2011).

Tadpole morphology was marginally affected by block (Pseudo-$F_{1,190}$ = 2.12, p = 0.086) and predator cues (Pseudo-$F_{1,190}$ = 2.25, p = 0.075) and significantly affected by developmental stage (Pseudo-$F_{3,190}$ = 1.25, p = 0.0234) and the Bd-by-predator-by-block interaction (Pseudo-$F_{1,190}$ = 2.54, p = 0.049). For the interaction, pairwise contrasts showed that within each block, these interactions were not significant (all p > 0.1). The marginally significant effect of predator caused tadpoles tail tips to curve ventrally and for the body to twist rostrally on the dorsal side and caudally on the ventral side, such that the mouthparts were shifted ventrally (Figure 5.5).

The tolerance model showed that predator cues significantly affected tadpole morphology (Pseudo-$F_{1,169}$ = 3.02, p = 0.016) while block and developmental stages had marginally significant effects (Pseudo-$F_{1,169}$ = 2.51, p = 0.054, Pseudo-$F_{1,169}$ = 2.15, p = 0.081, respectively). The effects predator cues on morphology for this model are similar to the effects of predator cues in the exposure model, so no image of this effect is shown.

The resistance model showed no significant effects (all p > 0.15) of treatments on activity; however smaller sample sizes for this comparison lowered the power of this test.

In addition to running a geometric morphometric analysis on tadpole morphology we also measured the effects of block, predator and Bd-exposure on five specific tadpole traits: tail length, tail depth, muscle depth, body length and body depth. This test showed a significant interactive effect of predator-by-Bd-by-mass on tail depth, muscle depth, body length and body depth (all p < 0.05). However, after use of the Johnson-Neyman technique to identify the range of mass where significant pairwise effects occurred, we did not detect effects of treatments on tadpole morphology (all p > 0.05).

5.4.4 Tadpole growth, development and survival

Predator cues and Bd exposure did not affect mass. Analysis of the exposure model showed a significant predator-by-Bd interaction ($F_{1,\ 240}$ = 4.04, p = 0.046), however, after adjusting for multiple comparisons, there were no significant pairwise comparisons. There were no treatment effects on mass in either the resistance model and or the tolerance model (all p > 0.46).

Uninfected and infected tadpoles exposed to Bd were more developed than tadpoles not exposed to Bd (Figure 5.6). The exposure model showed significant effects of Bd exposure and block on tadpole development (Wald χ^2_1, p = 0.014; Wald χ^2_1, p < 0.001, respectively). No other

terms were significant in the model (all $p > 0.40$). The resistance model showed the same pattern seen in the exposure model; uninfected tadpoles that were exposed to Bd were significantly more developed than tadpoles that were not exposed to Bd (Wald χ^2_1, $p = 0.031$). No other terms were significant in this model (all $p > 0.14$). The infection model showed that for Bd-exposed tadpoles, differences in development were not due to variation infection status; no terms were significant in this model (all $p > 0.094$).

There were no main or interactive effects of predator cues, Bd-exposure or block on the hazard of death ($p > 0.3$). Across all treatments, only 8% of the tadpoles died.

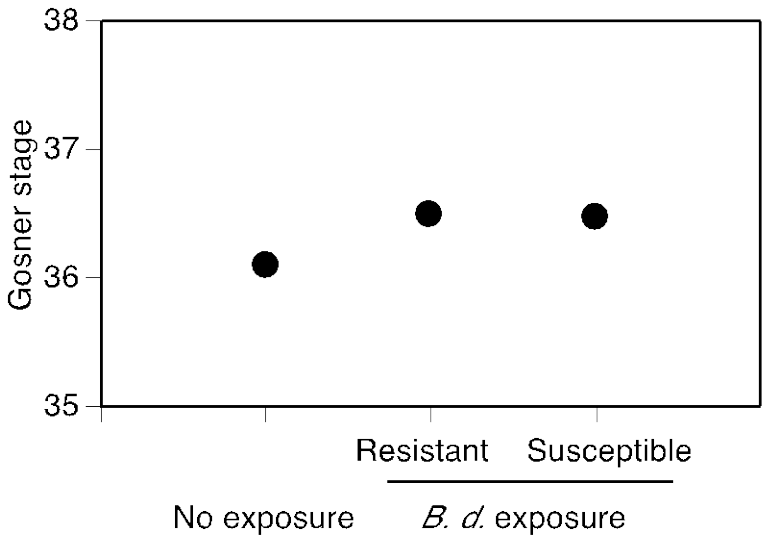


Figure 5.6. Effects of 23 d of exposure to Bd on the development of wood frog tadpoles.

5.5 DISCUSSION

The goal of this experiment was to test if exposure to predators altered infection prevalence and intensity and if exposure to pathogens altered inducible defenses against predators. We found evidence for both effects. Exposure to predator cues caused a decrease in pathogen load in prey that contracted an infection, but it did not alter the overall infection prevalence. Exposure to pathogens altered behavioral, but not morphological inducible defenses against predators. In addition, for the behavioral changes, the effect of pathogens on trait induction depended upon whether the tadpoles could resist infection. These behavioral changes would be predicted to decrease infection prevalence in tadpole populations because infected tadpoles were most active overall, making them most susceptible to predation, while resistant tadpoles were the least active. The model system used for this experiment included the fungal pathogen that causes the emerging infectious disease chytridiomycosis. These results suggest that predators should reduce infection prevalence and intensity in a suspected reservoir host by causing stress-induced immunoenhancement and because infection causes tadpoles to be more active, making them more susceptible to predation.

Our first hypothesis was that the stress of exposure to predators would alter the incidence and intensity of Bd infections. While predator cues did not alter Bd prevalence, they did cause infected tadpoles to have weaker infections at the end of the experiment. This result is consistent with past observations of risk reduction as a result of predator cues; wood frog metamorphs exposed to predator cues as tadpoles experienced a reduced hazard of death when exposed to Bd (chapter 3). If the same mechanism is driving both patterns, then the delayed mortality in Bd-exposed metamorphs may have been the result of lower infection intensity relative to tadpoles not exposed to predator cues, since virulence is often associated with

pathogen load (e.g. Vredenberg et al. 2010, but see Blaustein et al. 2005, reviewed in Fisher et al. 2009).

It is likely that the effect of predator cues on Bd loads resulted from stress-induced immunoenhancement. While predator-cues could alter behavioral traits associated with Bd resistance such as avoidance and behavioral fever (e. g. Han et al. 2008), the design of this experiment did not allow tadpoles opportunities to effectively express these behaviors. Behavioral avoidance was unlikely because the experimental units were small (1 L) and the concentration of Bd zoospores was quite high. Behavioral fever was not possible because there were no thermal gradients in experimental units. Thus, changes in infection intensity between treatments are most likely due to altered immune function.

Immune defenses of tadpoles against Bd infection are not well understood. For adult frogs, evidence suggests that both acquired and innate immune functions are important in fighting infections (reviewed in Rollins-Smith and Conlon 2005, Richmond et al. 2009, Ramsey et al. 2010, Voyles et al. 2010, Rollins-Smith et al. 2011). While stress often causes immunosuppression (reviewed in Apanius 1998), immunoenhancement resulting from stress has been observed for cell-mediated, humoral and innate immunity (reviewed in Dhabhar et al. 2000, Martin 2009). Moreover, stress-induced enhancement of skin immune functions (where Bd infection occurs) has been observed before and is thought to occur because stressful situations frequently result in exposure of the skin to harmful pathogens (Dhabhar et al. 2000). Identification of immune functions that are enhanced by predator-cues would aid in trying to understand intraspecific variation in tadpole susceptibility to Bd (Blaustein et al. 2005).

Our second hypothesis was that exposure to Bd would reduce induction of morphological and behavioral defenses against predators. We found that Bd altered tadpole behavior, but not

tadpole morphology. The effect of Bd-exposure on activity would be predicted to cause predators to select for Bd-resistant tadpoles. Infected tadpoles had increased activity levels, but only 8 d after exposure to Bd. Tadpoles that were uninfected at the end of the experiment were less active than controls, but this was only 15 d after exposure. Since activity increases risk of predation for these sit-and-wait predators (Skelly 1994), predators would be expected to consume proportionally more infected tadpoles from populations, and fewer tadpoles that have cleared or resisted infection.

Several other studies have examined the effects of Bd infection on tadpole activity, with a variety of results. Parris et al. (2006) found that infection with Bd caused *Rana pipiens* tadpoles to have reduced activity in the presence of bluegill predators. These tadpoles also were predated upon less than uninfected tadpoles. Similarly, Veneskey et al. (2009) found reduced larval foraging activity in Fowler's toads (*Anaxyrus fowleri*) that tested positive for an infection, but not in tadpoles that were exposed to Bd, but not infected. In contrast, Han (2011) found that exposure to Bd did not affect activity levels of *Rana aurora*, *Psuedacris regilla* or *Rana cascadae*, but it did cause *Anaxyrus boreas* to increase activity. For these studies, it is unclear whether different responses to Bd infection are due interspecific differences in costs of infection, differences in infection intensity, differences in the timing of measurement relative to the timing of *Bd* exposure or some combination of these traits. A possible explanation for the increased activity in infected tadpoles in this experiment that more foraging was required to maintain the same growth rate. Past studies have shown that Bd infections in mouthparts decrease feeding efficiency, so the extra activity may have been compensation for this cost and any other costs of an infection (Venesky et al. 2009, 2010). For uninfected, Bd-exposed tadpoles, reduced activity may have resulted from metabolically demanding immune responses, however, we did not take

the immune measurements to test this hypothesis. The results of this experiment suggest that caution should be made against drawing too many conclusions from a single snapshot of activity measurements since these effects are context- and time- dependent.

We did not see strong effects of exposure to predators on activity levels of tadpoles until later in the experiment, when we increased the concentration of predator cues and began measuring activity soon after applying predator cues. The lack of a predator effect early in the experiment is either because timing of activity measurements was delayed for too long after the application of predator cues or because the predator cues were not concentrated enough to induce activity changes. We believe that the former is the most likely scenario, since we saw effects of predators on morphological traits, which would be unlikely to change in the 3 d of increased predator cues that were applied at the end of the experiment. Transient effects of predator cues on activity levels have been observed before (Relyea 2003b).

We also tested if exposure to Bd and infection with Bd would change morphological inducible defenses of tadpoles against predators; however we found that Bd exposure did not alter tadpole morphology, while predator cues did. The effect of predators on tadpole morphology observed in this experiment is somewhat different than induced morphological defenses observed in other experiments. While we did not observe alterations in tail and muscle depth, which are typical for many tadpoles exposed to predators (Van Buskirk and Relyea 1998, reviewed in Relyea 2003a), there was a change in tadpole shape, such that, in the presence of predators the tadpole was curved with the anterior and posterior ends shifted ventrally (Figure 5.5). It is possible that this shape change often accompanies predator-induced morphology, but could not be detected with the more commonly used alternative morphological analyses (e.g., Relyea 2001). Another possibility is that this change was the result of predator

induction in small. One of the few studies to use a geometric morphometric analysis of tadpole landmarks examined tadpole morphology across a gradient of predator densities in nature and did not see this pattern (Van Buskirk 2009).

We did not observe any costs of responding to predator cues or Bd-exposure in tadpoles. The lack of any costs of predator cues is consistent with past studies of the effects of caged predators on development and growth of tadpoles. In a review of these effects, caged predators did not alter growth or development in slightly more than 50% of the experiments and reduced development or increased growth in 30-40% of the experiments (Relyea 2007). Costs of Bd infection in tadpoles have been shown for some species, but not others. For example, Blaustein et al. (2005) found that *Rana catesbeiana, Pseudacris regilla* and *Rana casacdae* could harbor Bd infections with few apparent costs, but *Bufo boreas*, a species which is declining rapidly in the wild (Wente et al. 2004) exhibited extreme sickness behavior and mortality.

In this study, both infected and uninfected Bd-exposed tadpoles were slightly more developed than unexposed tadpoles. Faster development is often associated with increased fitness (Howard and Kluge 1985, Smith 1987, Semlitsch et al. 1988); however, since post-metamorphic juvenile frogs often experience much higher Bd mortality, this increased development may in fact be a quicker walk to an infection. The lack of a cost of Bd infection for tadpoles is consistent with the idea that tadpoles may serve as a reservoir for Bd infection.

5.6 CONCLUSION

While traditional studies focus on the negative consequences of stressors, it is becoming increasingly clear that in some situations, exposure to threatening situations can have long-term

benefits (e. g. risk reduction; Sih et al. 1998, Romero 2004, Martin et al. 2009) The motivation for this study was to understand how two stressful situations, exposure to pathogens and predators change traits in the prey or host that alter their susceptibility to a second threat. In this situation we found that both stressors would result benefit exposed populations, though they might not benefit the exposed individual. Exposure to predators resulted in reduced risk of high levels of Bd infection, likely as a result of stress-induced immunoenhancement. Infection with Bd caused risk enhancement in the presence of predators while resistance to Bd caused risk reduction. Thus, while infected tadpoles were more likely to get eaten, their removal likely benefitted the population, by reducing opportunities for pathogen transmission. These results demonstrate that TMIIs can be very important for determining how pathogens will be regulated as a result of community-level interactions. These data suggest that TMIIs should receive increased consideration in studies of disease regulation in regulated in different environmental contexts. Moreover, mechanistic studies that can help determine when an additional stressor causes risk reduction or enhancement will be useful in developing more predictive models of when multiple stressors should interact to increase or decrease the population viability of host and pathogen populations.

6.0 CONCLUSIONS

This thesis examined how multiple stressors separately and interactively affect amphibian survival and health. This work was motivated both by ecological theory and by conservation concerns. The contribution of emerging infectious diseases to amphibian declines is large, and there is a critical need to understand the mechanisms underlying population-level variation in response to disease. These efforts are challenged because the fields of disease ecology and eco-immunology are at their infancy; we understand very little about how environmental heterogeneity influences within- and between- host pathogen dynamics or how pathogens influence population-, community- and ecosystem- level processes. To conclude this thesis I will highlight some of the contributions of this work to the growing fields of disease ecology and eco-immunology as well as to amphibian conservation.

The causes for amphibian population declines can be described as context-specific, interactive, and regionally variable. As such, it is extremely challenging to pinpoint if and when stressors will contribute to declines. They must be present at the right time and in the right context. Pesticides have been a suspected contributor to amphibian declines for some time; indeed many pesticides are highly toxic or immunosuppressive; however, the lack of any immunosuppressive effects of malathion in chapter 3 suggests that this is not always the case. Evidence (in chapter 4) that the timing and length of exposure to a stressor can dictate its effects suggests several contexts (e.g. early exposure or chronic exposure) through which malathion or

any stressor may be most deleterious. Identification of such windows where effects of stressors might be most profound will aid in the continued effort to understand the role of context-dependent, interactive stressors in amphibian population declines. Identification and use of such a window was perhaps most effectively done in chapter 5. By focusing our study on a suspected reservoir host of Bd, we were able to make broader conclusions about the importance of our findings (evidence for predator-regulated control of disease) for amphibian populations.

One of the more surprising patterns that came out of this work was how often exposure to stressors had positive individual- and population-level effects. This was seen in chapter 4, where production of antimicrobial peptides, a costly innate immune function, increased, despite a decrease in per capita resources to invest towards this function (e.g. competition). A rather different example occurred in chapter 3, where exposure to predator-cues increased survival later in life. A positive effect of predator cues was also seen in chapter 5 where exposure to predator-cues caused tadpoles to have lower infection loads. The idea that a single stressor can have both positive and negative effects on both short and long time scales is receiving increased attention, especially in the case of stress-induced immunoenhancement. Future challenges will be to understand the net effects of these co-occuring time- and pathway- dependent effects as well as the neuroendocrinological and neuroimmunological underpinnings of these effects. In some cases, the mechanism may be less molecular. For example, chapter 4 demonstrated that some of the variation in immunological function in response to stressors could be understood in the context of life-history theory. Tadpoles that delayed metamorphosis, had a greater time to recover from stressors they were exposed to and metamorphosed into more immunocompetent adults.

A major goal of this work was to make the case that trait-mediated indirect effects need increased consideration in understanding the impacts of pathogens in a community context. These effects are frequently ignored or underrepresented in the disease ecology literature, in large part because the empirical framework for these questions are based on models that frequently exclude these effects. Chapter 5 in particular demonstrated that these traits are important. Both predator-cues and exposure to Bd altered tadpole traits in ways that would be predicted to decrease the overall prevalence and intensity of Bd in a population. An important next step to this research is to understand how trait-mediated indirect effects of predators and pathogens interact with density-mediated indirect effects and to develop predictive models for when trait- or density- mediated effects should dominate interactions between hosts, pathogens and other community members.

In the future I hope to continue to understand how environmental stressors alter within- and between- host dynamics of pathogens and how pathogens alter the effects of environmental stressors. Technological advances, increased interest from funding agencies and compelling research and debate on these subjects make this an exciting time to be in this field.

APPENDIX A

SUMMARY OF WOODFROG ANTIMICROBIAL PEPTIDES

A.1 WOOD FROG PEPTIDES

Table A.1 Peptides found in wood frog metamorphs collected as eggs in Linesville, PA. Peptide sequences were assigned manually to spectra collected using nano-flow electrospray liquid chromatography quadrapole time-of-flight tandem mass spectrometry. Other temporins, including the most similar temporin and the temporin concensus sequence are also shown.

Peptide name	**Organism**	**Molecular weight (Monoisotopic)**	**Proposed sequence**	**Source**
Brevinin-1SY	*R. sylvatica*	2440.3	FLPVVAGLAAKVIPSIICAVTKCC	Mattute et al. 2000
Temporin-1SY	*R. sylvatica*	1521.8	(L/I)(L/I)FSA(L/I)GNA(L/I)SR(L/I)F-NH_2	This study
Peptide A1	*R. esculenta*	1389	FLPAIAGILSQLF	Simmaco et al. 1990
Temporin-Prb	*R. pretiosa*	1391	LLPIVGNLLKSLL-NH_2	Simmaco et al. 1996
Temporin consensus sequence (1)		1382	FLPILGSLLS(G/K)LL-NH_2	Wade 2010
Temporin consensus Sequence (2)		1412	FLPI(L/I)G(S/K)LLSGLL-NH_2	Wade 2010

A.2 MASS SPECTROMETRY

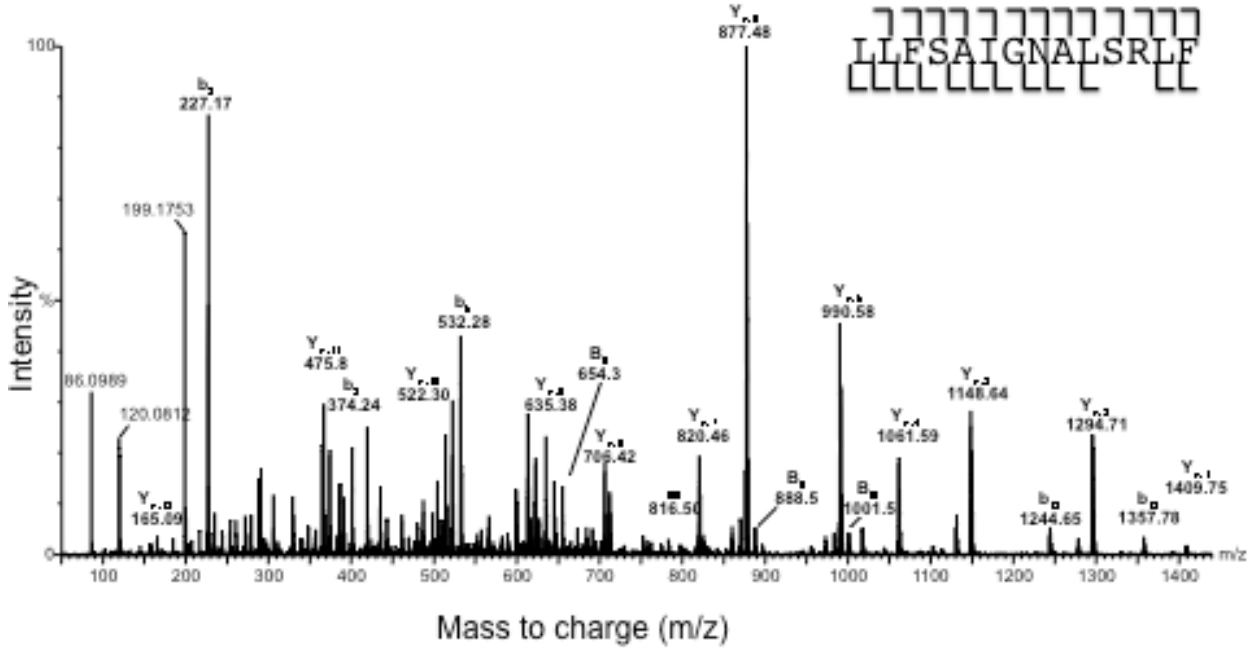


Figure A.1 Tandem mass spectrum for temporin-1SY (molecular weight 1521.8) acquired with nano-flow electro-spray liquid chromatography quadrapole tandem mass spectrometry (Q-TOF II ESI/APCI Quadrupole-TOF, Waters Corporation). Isoleucine and leucine in this spectra were assigned to maximize homology with similar sequences.

APPENDIX B

SUMMARY OF LEOPARD FROG ANTIMICROBIAL PEPTIDES

B.1 LEOPARD FROG PEPTIDES

Table B.1 Brevinins found in leopard frogs and partial sequences for suspected brevinins found in this paper. Assignment of leucine and isoleucine was done to maximize homology with similar peptides. In some cases, both leucine and isoleucine seemed equally likely. These instances are indicated with '!'.

Sequence name	Molecular weight (Monoisotopic)	Sequence	Citation
Likely New	1875.2	FLPIVV!VPF!!...	This study
Likely Brevinin-1Pd	2569.6	FLPIIASVA…	This study
Likely New	2593.9	FFP!VAR…	This study

Table B.1 Continued

Likely New	2623.9	FFPNVASV…	This study
Likely new	2877.0	FFP!VA…	This study
Brevinin-1Pa	2561.4	FLPIIAGVAAKVFPKIFCAISKKC	Horikawa et al. 1985; Goraya et al. 2000; Tennessen and Blouin 2007, 2008
Brevinin-1Pb	2575.4	FLPIIAGIAAKVFPKIFCAISKKC	Horikawa et al. 1985; Goraya et al. 2000; Tennessen and Blouin 2007
Brevinin-1Pc	2581.5	FLPIIASVAAKVFSKIFCAISKKC	Horikawa et al. 1985; Goraya et al. 2000
Brevinin-1Pd	2567.5	FLPIIASVAANVFSKIFCAISKKC	Goraya et al. 2000
Brevinin-1Pe	2591.5	FLPIIASVAAKVFPKIFCAISKKC	Goraya et al. 2000; Tennessen and Blouin 2007
Brevinin-1Pf	2589.5	FLPIIAGIAAKFLPKIFCAISKKC	Tennessen and Blouin 2007
Brevinin-1Pg	2594.5	FFPIVAGVAGQVLKKIFCTISKKC	Tennessen and Blouin 2007, 2008
Brevinin-1Ph	2543.3	GIPLLPGLAANLCRPIYCTITKNC	Tennessen and Blouin 2007
Brevinin-1Pi	1834.0	GIPLLPGLAANLCRPINC	Tennessen and Blouin 2007

Table B.1 Continued

Brevinin-1Pj	2651.4	FFPNVASVPGQVLRKIFCAISKKC	Tennessen and Blouin 2008
Brevinin-1Pk	2593.5	FLPIIAGVAAKVFPKIFCTISKKC	Tennessen and Blouin 2008
Brevinin-1Pl	2607.4	FLPIIAGMAAKFLPKIFCAISKKC	Tennesseen et al. 2010

Table B.2 Temporins closely resembling the temporin found in this paper. The other temporin identified in *Rana pipiens* is also shown. Assignment of isoleucine and leucine in Temporin-2P is done to maximize homology with known sequences. Cases where an assignment could not be made are indicated with an '!'.

Sequence Name	**Monoisotopic Molecular Weight**	**Host species**	**Sequence**	**Citation**
Temporin-2P	1427.1	*Rana pipiens*	FLPIVNA!!SG!NG	This paper
Temporin-CPb	1395.8	*Lithobates capito*	FLPIVGRLISGIL	Conlon et al. 2009
Temporin-1ARa	1397	*Rana aerolata*	FLPIVGRLISGLL	Ali et al. 2002
Temporin-1VE	1368.8	*Rana versabilis*	FLPLVGKILSGLI	Chen et al. 2006
Temporin-1PLa	1368.8	*Rana palustris*	FLPLVGKILSGLI	Basir et al. 2005
Temporin-1P	1368.1	*Rana pipiens*	FLPIVGKLLSGLL	Goraya et al. 2000
Temporin-1M	1368.1	*Rana muscosa*	FLPIVGKLLSGLL	Rollins-Smith et al. 2006.
Temporin-SHb	1464.8	*Pelophylax saharica*	FLPIVTNLLSGLL	Abbassi et al. 2008

B.2 MASS SPECTROMETRY

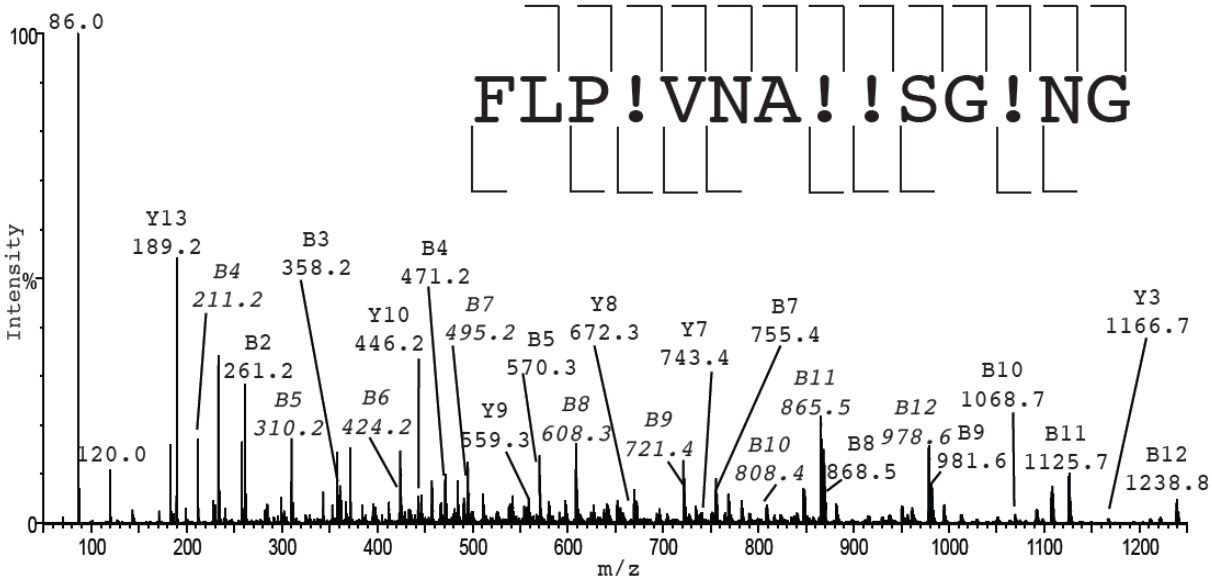
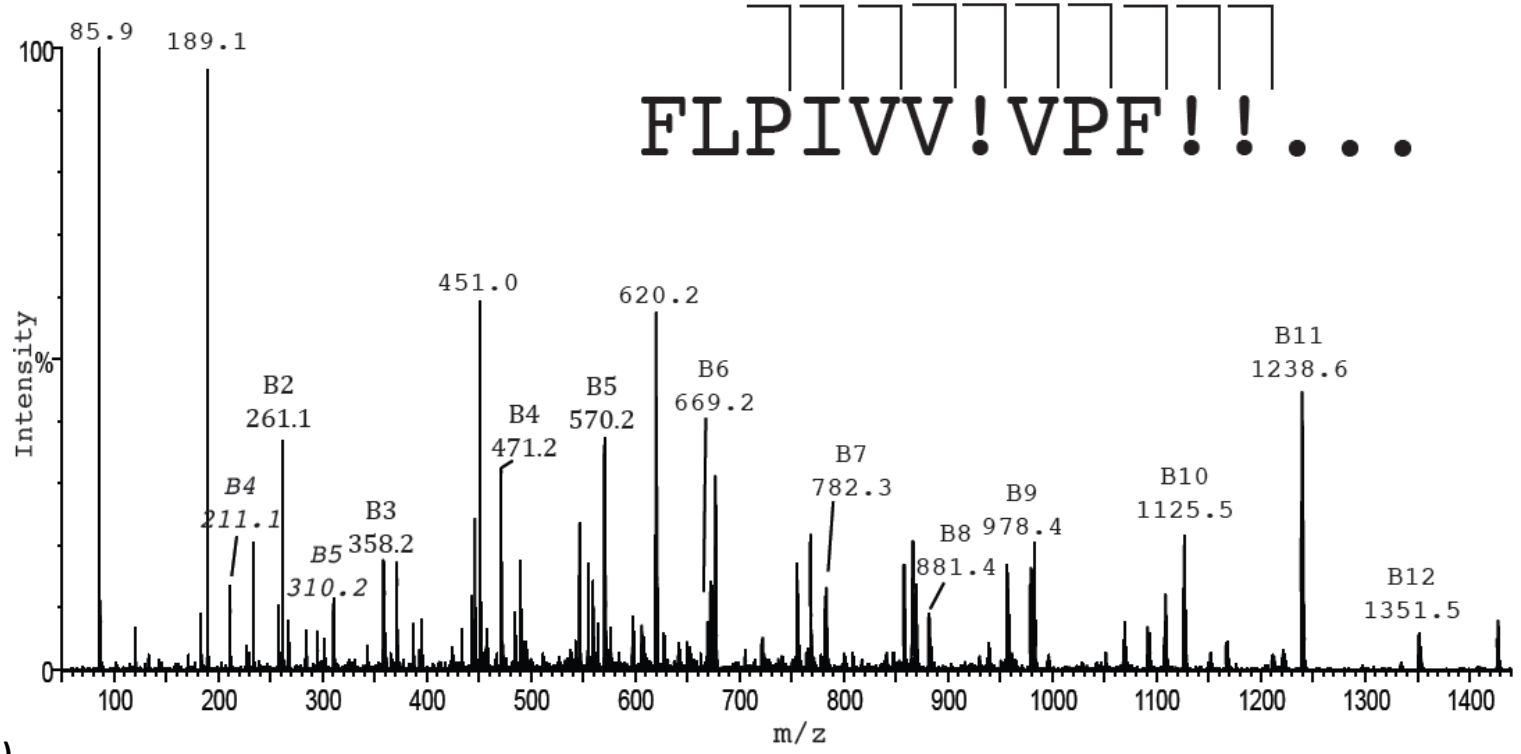


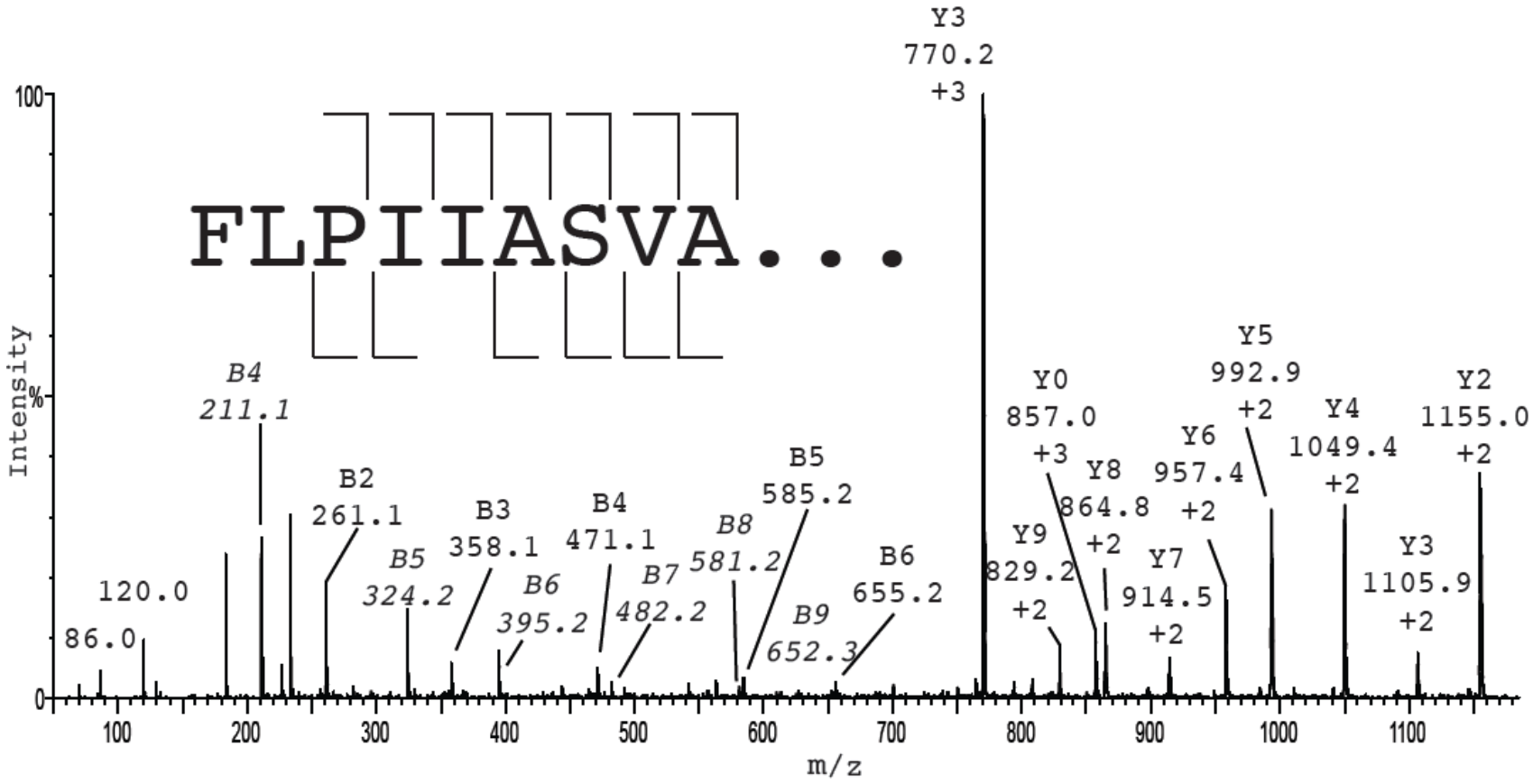
Figure B.1 Tandem mass spectra for temporin-2P acquired with nano-flow electro-spray liquid chromatography quadrapole tandem mass spectrometry (Q-TOF II ESI/APCI Quadrupole-TOF, Waters Corporation). The sequence interpreted is also shown with bars above indicating support for characterization from b-series ions and bars below indicating support for characterization from y-series ions. Proline in the third position gave rise to a second b-series beginning with that residue. The latter b-series is italicized. Assignment of leucine and isoleucine was done to maximize homology with similar peptides. Cases where an assignment could not be made are are noted as '!'.



A)

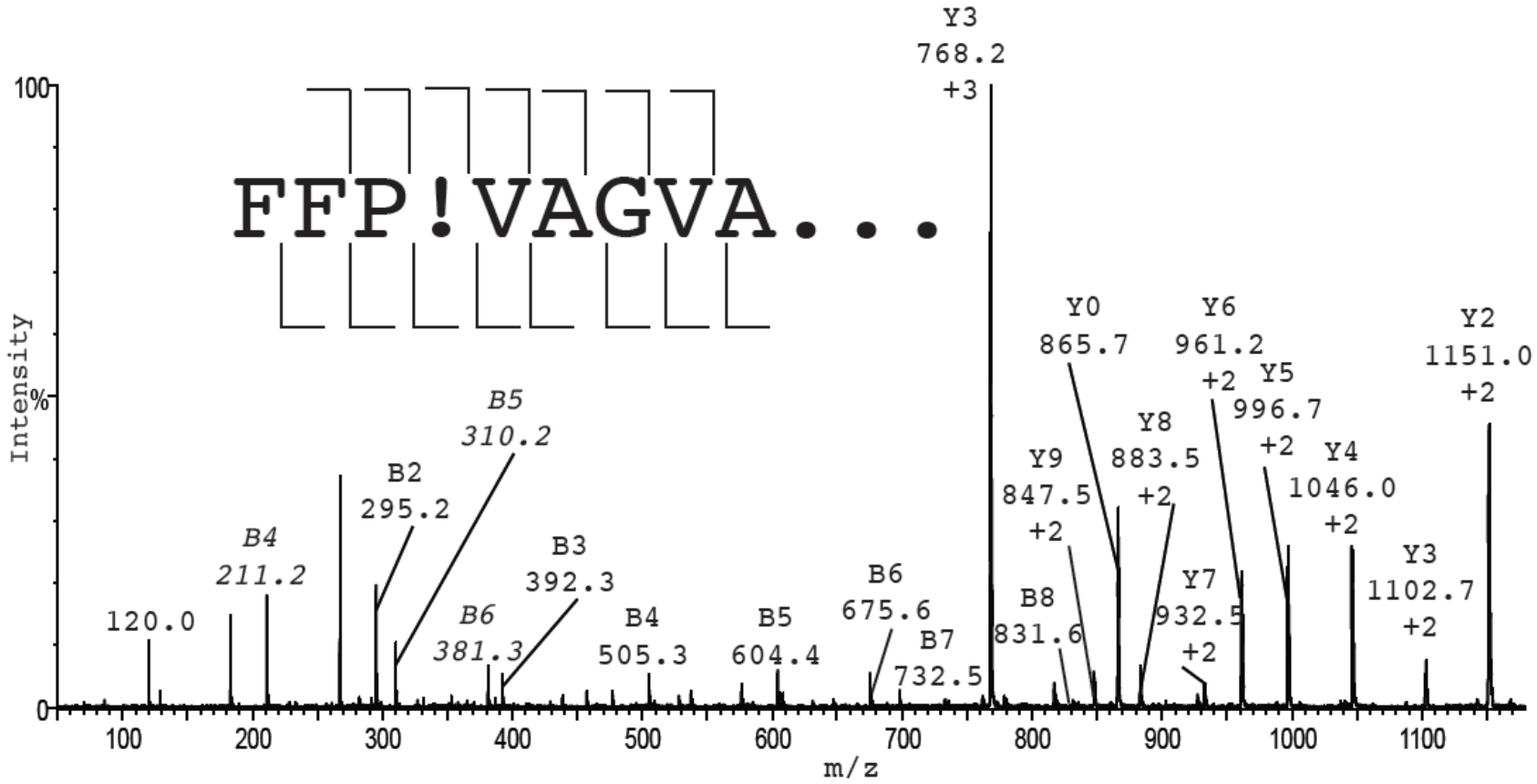
Figure B.2 Tandem mass spectra for potential brevinins acquired with nano-flow electro-spray liquid chromatography quadrapole tandem mass spectrometry (Q-TOF II ESI/APCI Quadrupole-TOF, Waters Corporation). Molecular weights of spectra are a) 1875.2, b) 2569.6, c) 2593.9, d) 2623.9, and e) 2877.0. The sequence interpreted is also shown with bars above indicating support for characterization from b-series ions and bars below indicating support for characterization from y-series ions. For most of these spectra, a proline in the third position caused the b-series to cleave after B2. As a result, 2 b-series were detected, on beginning with the dipeptide for B1 and B2 and the other beginning with the dipeptide corresponding to B3 and B4. The latter is italicized. Assignment of leucine and isoleucine was done to maximize homology with similar peptides. Cases where an assignment could not be made are are noted as '!'.

Figure B.2 (Continued)



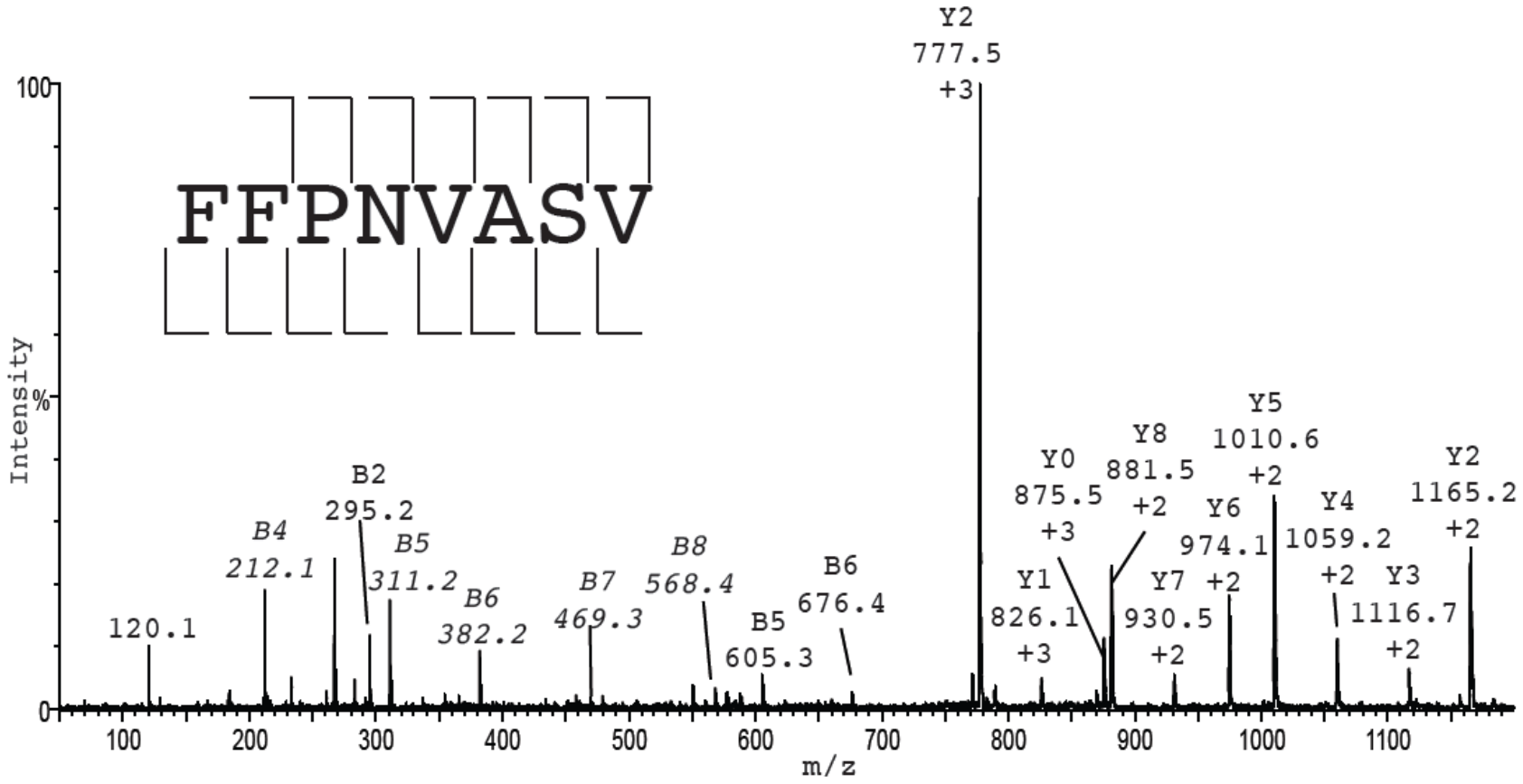
B)

Figure B.2 (Continued)



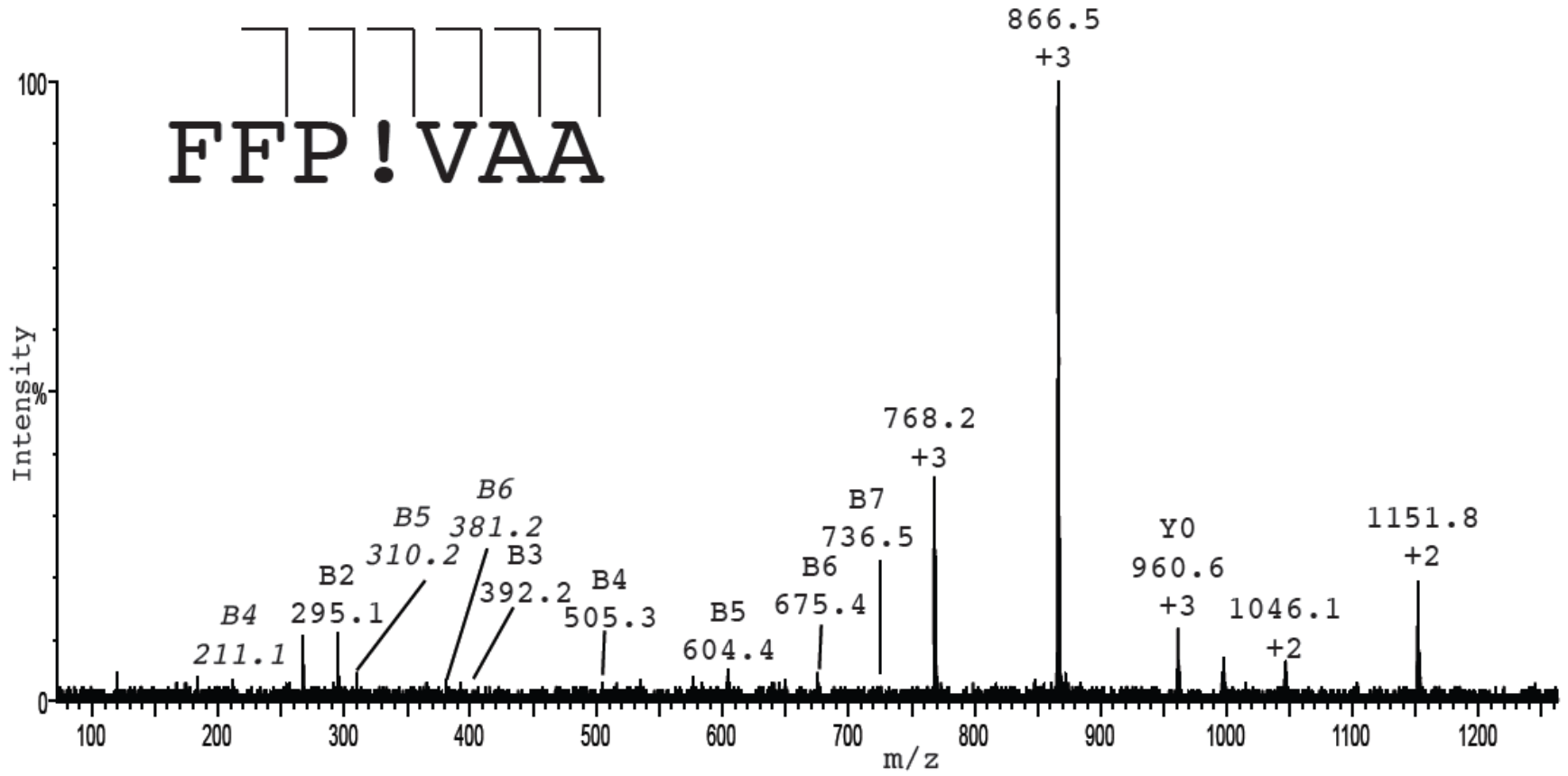
C)

Figure B.2 (Continued)



D)

Figure B.2 (Continued)


FFP!VAA
Intensity
%
100
0
866.5
+3
768.2
+3
B7
736.5
B6
675.4
B5
604.4
B4
505.3
B3
392.2
B6
381.2
B5
310.2
B2
295.1
B4
211.1
Y0
960.6
+3
1046.1
+2
1151.8
+2
100
200
300
400
500
600
700
800
900
1000
1100
1200
m/z

E)

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